



13 December 1979

Science in schools: progress barely satisfactory

IT IS NOT an entirely cheerful picture of science education at the secondary level which emerges from a report just published by Britain's Department of Education and Science (*Aspects of Secondary Education in England*: HMSO, £6.75). The Schools Inspectorate has been examining the performance of one tenth of all the secondary schools in England over the period 1975-1978. Of these 384 schools, 148 were still classified as grammar or secondary modern — the remainder were comprehensive schools of one sort or another. The inspectorate concentrated on the 4th and 5th years, the last two years before the majority of pupils leave and the remainder begin to focus more intensively on their specialist choice. And science was one of the subjects to which special attention was directed.

It is hardly surprising that within a range of nearly four hundred schools quite extraordinary diversity should be found in the provisions made for pupils; at one extreme a large school offered eight different science courses; at the other extreme a much smaller one, for girls only, offered just optional biology. Less than 20% of all schools have a science subject in their core curriculum of compulsory subjects and indeed 9% of all boys and 17% of all girls do no science at all. The numbers who only do one science subject are 50% and 60% respectively.

Even though there have been significant changes in attitude in recent years, the tradition that boys do physical sciences whilst girls do biology still prevails. 48% of all boys study physics and 31% study biology, whereas the figures for girls are 10% and 58% respectively. Some teachers claimed that physics was too difficult for girls, whilst in certain schools, the timetable for options was such that physics was set against a subject usually regarded as for girls — for example, home economics or typing. And there is fairly clear evidence for the often-quoted assertion that girls in single-sex schools turn more readily to physics than they do in mixed schools.

The qualifications of the teachers are, on the whole, appropriate to the subjects they teach in chemistry and biology. But many physics teachers lack any qualification in the subject: one in six did not specialise in physics but in some other science or

mathematics, whilst one in twenty has no scientific qualification at all. Whereas there are doubtless many excellent teachers in this group, the inspectors remark that in at least 10% of schools, one or more science teachers "did not know the subject sufficiently well and false information was taught".

It is to the style of teaching that most criticism is aimed, however. In around one-thirds of all schools the teaching was "always or nearly always overdirected, with insufficient pupil activity". Particularly in biology classes pupils were expected to take copious notes from the black-board or by dictation, leaving little scope for individual thought. And in all subjects, the doing of experiments was too closely tied to there being a right answer that had to be obtained; experimentation was not often regarded as something open-ended that pays dividends to those who are observant. The idea of science as a process, rather than purely as the contents of textbooks is still rarely instilled in young people.

Not that textbooks are in too plentiful supply. In more than half of all schools the extent to which books were available and used was 'disappointing'. The problem was particularly marked in the case of less-able pupils. Rarely were pupils encouraged to develop reference skills by consulting a range of books and other learning aids. Further, external resources such as industry, museums and field centres were generally thought to be underused. And teachers had given little consideration to how the scientific methods might impinge on other subjects, from geography to metalwork.

Financial constraints can no doubt be blamed for many of the problems that have been turned up. But the foreseeable future is likely to bring little relief in this direction, whilst it most certainly will bring fewer new recruits to the profession. So any steps forward have to make do with limited funds and the teaching profession as it now is. This means a concerted effort to provide much more in-service training than at present, not just to hand out extra information for those teachers whose background or formal education is now no longer totally adequate, but also to provide new horizons, such as science-as-a-process, science in industry, and the scientific approach in other disciplines □



US biomedical research faces financial stringency

After five years of sustained growth, US biomedical research is once again about to face a period of austerity. **David Dickson** reports.

Since 1975, the growth of support for biomedical research in the US has outstripped virtually all other areas of the science budget. But fiscal stringency and changing social priorities are bringing this to an end and biomedical scientists will soon face some hard decisions as they adjust to the budgetary austerity destined to characterise the early 1980s.

The Administration has already adopted "stable funding" as the cornerstone to its strategy and in its budget request for the fiscal year 1981, due to be presented to Congress by President Carter next month, "stabilisation" is expected to be interpreted as the need to maintain the number of new grants for investigator-initiated research projects at its current level of about five thousand per year.

By doing this, the Administration hopes to provide some protection for the "science base" of its health research programme, steering public discussion of resource allocation to areas more directly related to specific health problems.

Research planners are worried, however, that unless this form of stabilisation is accompanied by an adequate growth in the overall budget, it could lead to serious difficulties in other areas of the science base — such as the intramural research of the National Institutes of Health, or its support for research centres.

Last week Dr Donald Fredrickson, director of NIH, held a meeting of his advisory committee in Bethesda to discuss the implications of a stabilisation strategy based on a low rate of growth. Introducing the meeting, he made it clear that budgetary stringency increased the need for the institutes to be "in the best posture to react to congressional action".

He listed three external sources of pressure to which NIH was having to respond. The first is a process initiated last year by Mr Joseph Califano, then Secretary for Health, Education and Welfare, aimed at placing health research planning — including support for fundamental research related to health applications — on a five-year basis.

The second source of pressure is a bill introduced into Congress earlier this year by Senator Edward Kennedy proposing, among other things, a presidential commission for health sciences, which would be responsible for recommending budgets and research priorities over a five-year period. The third is the imminent announcement of the President's 1981 budget request.

Of the three, it is the last which is



Fredrickson: preparing NIH for congressional action

currently receiving the closest attention within the Administration. In the five years 1974 to 1979, the research budget of DHEW, over 90% of which passes through NIH, increased from \$2 to \$3 billion, an average increase of 10% a year, considerably higher than the science budgets of most other federal agencies.

But the rate of growth has now slackened. In his 1980 budget request, President Carter initially proposed no increase for biomedical research. As expected, this was subsequently changed by Congress, which voted an 8%.

The precise budget figures for 1981 are still under discussion. However there are indications that, as last year, the President may ask for virtually no increase in funding — and that Congress will be even more reluctant to add to the request.

As for funding priorities, Dr Gilbert Omenn, Associate Director of the Office of Science and Technology Policy, told the members of the advisory committee that the Administration was proposing to emphasise the desirability of keeping up the number of competitive research grants, even if this meant putting additional pressure on the rest of the system.

"We are intending to protect the individual research awards against the type of encroachment that took place in the early 1970s" Dr Omenn said. Stabilisation of research funding was not an ideal solution, but it was a pragmatic approach to the present climate.

Earlier in the day, however, several speakers described the potential consequences of stabilising competitive grants without at the same time ensuring an adequate level of overall support. Dr William Raub, for example, Associate Director of NIH for Intramural Research, said that if the number of competing grants were kept constant but there was no net increase in the budget, funding for other activities supported by NIH could drop 50% over a five-year period.

Details of how a budget squeeze could

affect the intramural programmes of NIH were described by Dr Robert Goldberger, recently appointed Deputy Director for Science. In 1979, mandatory costs such as salaries absorbed 72% of the intramural budget. Even with a 7% budget increase in 1981, NIH would theoretically have to reduce its workforce by about 14% if it chose this way to maintain the same balance between mandatory and other costs, he said.

Also affected by a budget squeeze would be the National Library of Medicine. Dr Martin Cumming, Director of the Library, told the committee that the inflation rate for libraries was running at about twice the national inflation rate, and that current funding levels made it very difficult to maintain a fully comprehensive service.

Having identified the possible hazards ahead, Dr Fredrickson reminded the members of the committee that, however antagonistic scientists might be to attempts to forecast future research needs and priorities, some attempt at rationalising the total effort was needed to gain congressional support.

Congress had recently wearied of trying to provide adequate support for research into each disease — what was now needed was to convince legislators to guarantee support for a research floor. "The word 'floor' is political, because we all know that we can do more quality science than we can support. The word 'floor' is trying to convince people that there should be a minimum," Dr Fredrickson said. Mr Califano has already committed the Administration to seeking ways of ensuring stable and predictable funding for biomedical research, arguing in particular that a five-year forward plan would help protect research funding from short-term political considerations.

Mr Califano's proposals received a relatively rough ride when they were debated by the scientific community at a conference held at NIH in October last year. Many speakers insisted that the prime need was for more money, not a different type of management.

More enthusiastic support, however, subsequently came from the Institute of Medicine. A panel of the institute came out in favour of long-range planning, arguing that it offered the "best assurance that there will be optimal allocation of resources for health research".

The IoM report, whose main conclusions were outlined to Dr Fredrickson's advisory committee by institute president Dr David Hamburg, endorsed the idea of a five-year plan for health services research. And to help preserve long-term stability of funding, it suggested setting up a permanent mechanism for regularly reviewing and updating the plan, a recommendation subsequently quoted by Senator Kennedy

in support of his own proposal for a presidential commission.

At the NIH there is considerable concern about such proposals. Many fear the implication that it might remove from the institutes responsibility for deciding how basic research funds should be allocated. In terms strongly reminiscent of the debate in Britain that followed the proposals made by Lord Rothschild for reorganising medical research in the early 1970s, they argue that planning is only effective when it is done by those with responsibility for carrying out the resultant decisions.

Dr Fredrickson's reluctance to see the NIH lose its grip on setting scientific priorities was endorsed by several advisory committee members. "In today's world it is often desirable to have a document available that you can refer to as your plan. But the greatest value of such a document is in its preparation — often the plan itself is best forgotten once it has been conceived" said Mr Benno C Schmidt, previously chairman of the President's cancer commission.

Other speakers recommended that if there were to be outside advice provided to government policy-makers, it should focus on management issues, rather than on the disaggregation of scientific priorities. "In five years as a member of the Defense

Science Board, I can remember only having discussed a scientific issue once. In the vast majority of cases we were involved with ways of helping the Department of Defense manage its resources better," said Dr Ivan Bennett, Provost and Dean of the New York University Medical Center.

Yet the issue of public participation is unlikely to be resolved that easily. One of the most controversial sections of the bill proposed by Senator Kennedy, due to be voted on by the Human Resources Committee in the next few weeks, is public participation in the peer review process, an idea anathema to most working scientists.

Mr Califano has previously expressed DHEW's strong opposition to the bill. Yet among the principles on which he has said health research should be established, it is stated that "to assure that HEW health research is responsive to public concerns, the public must participate in the setting of research policies and priorities".

So far, public participation has been interpreted largely as public comment, rather than significant involvement in decision-making.

"The process of public participation has been highly successful numerically, but not so effective in terms of give and take," said Dr Omenn. Despite the amount of material, there had not been much cross-

cutting between people with a stake in the current system, and those with other ideas.

There will be a further opportunity for public debate soon when DHEW publishes a set of possible research initiatives for 1980 to 1984. These have been prepared by a steering committee under Dr Fredrickson as an attempt to convert Mr Califano's "principles" into budget language. And they are expected to include "trans-NIH" programmes in subjects such as smoking and nutrition, as well as the suggested stabilisation of research project grants currently being pushed by OSTP.

The progress of next year's budget request through Congress will indicate how well these proposals, and the philosophy behind them, fare in the world of *real-politik*. Dr Omenn reminded committee members that there might soon be a change in command in the Senate appropriations subcommittee responsible for medical research and that among potential candidates was Mr William Proxmire, no great friend of the research community.

"The numbers that we are proposing can be argued about", he said. But a stabilising strategy was important because it was "the type of process that those people who may dominate the appropriations committees for the next few years will be able to agree on". □

Research on foot and mouth virus approved

In the first major decision on potentially hazardous experiments requiring special permission under federal safety guidelines, the recombinant DNA advisory committee of the US National Institutes of Health has given its approval in principle for experiments to be carried out on foot and mouth virus. Research workers hope that the research will eventually lead to the production of a new vaccine against the disease, a major threat to cattle in many parts of the world, and provide important information about antigenicity mechanisms.

The advisory committee last week recommended to the Director of NIH, Dr Donald Fredrickson, that he approve an application for such experiments that have been made jointly by scientists at the Plum Island Animal Disease Centre of the US Department of Agriculture — the only place in the US currently allowed to cultivate and study foot and mouth disease (FMD) virus — and the San Francisco firm, Genentech.

However before giving the experiments detailed approval, the committee has appointed a subcommittee to study the infectivity of the virus's genome and it is insisting that when genetic material from the virus is studied or transported, it should be in lengths short enough to ensure that there is virtually no danger of infection.

Under the NIH guidelines for recombinant DNA experiments, foot and mouth disease virus belongs to the set of

pathogenic organisms on which experiments are prohibited without the specific approval of the Director of NIH.

The research proposal is aimed at identifying that part of the genetic material which codes for a protein attached to the virus surface which, if produced and administered in sufficient quantities,

would make an effective vaccine. Current vaccines are made from the whole virus, and the production techniques necessary to produce the 800 million doses of the vaccine used annually throughout the world are thought to be partly responsible for some of the outbreaks of the disease.

Under the research proposal submitted to NIH, research workers on Plum Island will produce double-stranded DNA from the FMD virions. Plasmids containing subgenomic fragments of the DNA will be prepared using the disabled *E. coli* K12 strain of bacteria and, after suitable testing against infectivity, will be transferred to Genentech in San Francisco.

Here the fragments will be characterised to identify that part of the genome corresponding to the desired protein. Once this has been achieved the protein can be expressed in *E. coli*, and it will then be tested for antigenic and immunogenic potency on animals at Plum Island.

At its meeting last week, the advisory committee agreed that it was important that the experiments should be carried out for both economic and scientific reasons. Its main concern was to ensure that the genome was fragmented so that infection was virtually guaranteed to be impossible.

One way to do this, according to Dr Howard Bachrach, the principal investigator on Plum Island, was to divide the genome into two segments, the longer one of which research workers at Pirbright in England had shown to be non-infective. □

Carter sacks nuclear regulation chief

Following consideration of the report of the Kemeny commission on last March's accident at the Three Mile Island nuclear plant, President Carter announced last week that he was replacing Mr Joseph Hendrie as chairman of the Nuclear Regulatory Commission.

However the President said that the accident had not changed his views about the need for nuclear power. "We cannot shut the door on nuclear energy", he said. Recent events in Iran had shown the "clear, stark dangers" of excessive dependence on imported oil.

President Carter said that he agreed fully with the "spirit and intent" of the Kemeny commission's recommendations. However, he urged that the NRC should complete its current internal review — during which no new licenses or construction permits are being issued — "as quickly as possible," and in any event "no later than six months from today".

Short term tests can predict potential carcinogens, says study

RESULTS of a unique international study to evaluate tests for the detection of chemical carcinogens were released in London last week. They confirm that there are short-term tests that can be used to predict carcinogenic activity. No simple assay or battery of tests is readily apparent as best-suited for assessing carcinogenicity; however, the coordinating committee of the study believes that the day is nearer when such a battery can be assembled to screen out potential carcinogenic chemicals.

The international programme for the evaluation of short-term tests for carcinogenicity began three years ago as a joint venture by the UK Medical Research Council (MRC) and Health and Safety Executive. Scientists at ICI Central Toxicology Laboratory (CTL) were asked to participate and the programme was later joined by researchers at the US National Institute of Environmental Health Sciences (NIEHS), who were keen to broaden the scope of the study to the level of a truly international trial of assay systems. Additional support and finance for an expanded programme was solicited and eventually provided by the US Environmental Protection Agency and National Cancer Research Institute in Japan.

The aim of the study, according to the coordinating committee chairman Dr Frederick J de Serres of NIEHS, was to examine the performance of various short-term tests which have been used in attempts to distinguish between known carcinogens and known non-carcinogens. Although many of the thirty tests used in the study are based on the ability of bacterial, fungal or mammalian cells to mutate, de Serres was insistent that the tests under scrutiny were being evaluated as "predictors of carcinogenic activity" and not just as tests for examining the mutagenic properties of chemicals.

It is well known that short-term tests can be fickle, giving better results for one class of chemical than another. It is equally true that many of the tests evaluated so far have been studied with the researcher knowing both the identity of the chemical being tested and its carcinogenicity in animals. To eliminate this built-in bias the 60 or so laboratories participating in the international programme were sent coded samples, the identities of which were only revealed after investigators had submitted their final results. 42 chemicals, 25 carcinogens and 17 non-carcinogens from as wide a range of chemical classes as possible, were selected on the basis of data available concerning their carcinogenic properties in animals (see *Nature*, 274, 740, (1978)).

The results presented last week were a

summary of discussions of two meetings held in March and October of this year to assess the findings. On the basis of the data evaluated so far the coordinating committee concludes that the most effective tests are those using bacteria such as *Salmonella* and *Escherichia coli*. Tests using strains of *Salmonella* to detect mutagenic chemicals were first developed by Dr Bruce Ames at the University of California (the 'Ames' test). Although Ames personally declined to participate in the study, some twelve laboratories using this test did take part. The so-called 'Ames sub-set' of bacteria tests were found to be highly predictive of potential chemical carcinogens.

But the bacterial-based assay do not detect carcinogens in every case. No positive result is obtained for the potent carcinogen hexamethylphosphoramide (HMPA), whereas a positive result is obtained for both fungal assay systems using yeast and mammalian cell tests. All tests give such false negatives and false positives too; the committee concludes, therefore, that the most effective results can be obtained by using a battery of tests.

According to Dr John Ashby of CTL, the committee considers the bacterial-based tests to be the most reliable, but the mammalian cell tests are necessary to "fill in the holes"; and assays based on whole animal systems such as the sex-linked recessors lethal tests using *Drosophila* or the mouse-sister-chromatid exchange tests are 'good arbiters' of the other two, but are not yet ready to be "worked up into full test systems".

Professor Bryn Bridges of the MRC Cell Mutation Unit at Sussex University made the point that more research is required to study the nuts and bolts of the assay systems if short-term tests are to be used as a screening net in potential carcinogens. Because there is still much to be learned, Bridges warns against the temptation facing some government regulating authorities to lay down cast-iron rules for the application of tests. If this happens, says Bridges, it will 'fossilise' the tests and limit their effectiveness.

Aware of the commercial potential of their recommendations the coordinating committee is being deliberately cautious about recommending any one assay system. Many commercial laboratories, already involved in routine testing, could cash in on the findings of this study. It is too early, says the committee, to identify the appropriate battery of tests to be used on the basis of a study restricted to forty-two chemicals. Nevertheless, it is clear that the results of this study will have considerable influence on regulatory authorities. It will be published in full by June 1980.

Alastair Hay



Polish animal nutritionist refused passport

Dr Jan Kielanowski, (above) a member of the Polish Academy of Sciences and an acknowledged expert on pig rearing was last week refused a passport to Britain to chair a symposium on "Future perspectives in pig nutrition", organised by the British Nutrition Society. Dr Kielanowski is head of the Polish unofficial 'Society of Academic Courses' (the "Flying University" — see *Nature* 6 December, page 543). In May of this year, together with Dr Edward Lipinski, an economist, he attempted to bring before the General Assembly of the Polish Academy of Sciences a motion calling for the abolition of press censorship and greater academic liberty.

Yugoslavia changes nuclear site after protests

YUGOSLAV energy planners have decided to site Croatia's second nuclear power station at Prevlaka on the river Sava, near Zagreb. This decision follows concerted local protests against the site originally projected, the island of Vir near Zadar on the coast. Plans for a station on Vir have not, however, been abandoned. In all, four nuclear power stations with a total capacity of 3500 MW are to be built in Croatia within the next 20 years. Croatia has coal reserves for only thirty years and will have exhausted its hydroelectric potential by the end of the century.

Last July, R Pavlovic, director of the Croatia Electricity Board announced that even if the second station should be transferred to an inland site, all future nuclear power stations would have to be built on the coast, since only the sea can provide sufficient water for them. Last month's decision by the Croatian Association of Electric Power enterprises to build the second station at Prevlaka included a resolution to continue research work at Vir. Certain industrial interests in Croatia are anxious to develop along the coast some industry other than tourism, which they see, at best, as too dependent on factors external to the Yugoslav economy.

Vera Rich

news in brief

Dutch parliament rejects new nuclear missiles: The Dutch parliament voted last week against the NATO nuclear missile modernisation programme and against the stationing of the Cruise missile in Holland. The vote is expected to force the resignation of Prime Minister Dries van Agt unless he votes against the missile deployment at the NATO summit meeting this week in Brussels. Holland now joins Denmark in opposing the plan to place 500 intermediate range nuclear missiles on European soil. Belgium may also join the opponents of the plan as both the French and Dutch speaking socialist parties are opposed to it. The Italian Chamber of Deputies, however, voted last week by 328-230 in favour of the siting of the Pershing II and Cruise missiles in Italy.

Upton resigns as cancer institute head: Dr Arthur Upton announced last week that he was resigning his post as Director of the National Cancer Institute to become Director of the Institute of Environmental Medicine at New York University Medical School. Dr Upton has been Director of the NCI, whose projected budget for 1980 is more than \$1000 million, since 1977. He was previously on the faculty of the Health Science Center at the State University of New York at Stonybrook. Dr Vincent T Devita, head of the NCI's Division of Cancer Treatment, has been named acting head of the NCI until a replacement for Dr Upton is found.

During his term at NCI, Dr Upton has both placed greater emphasis on basic research, and on environmental and occupational causes of cancer. He said that he was resigning as an administrator because he missed "the more direct personal involvement in science".

Britain to shake up its engineering profession: A copy of the long awaited Finniston report (*Nature*, 2 August page 352) on the state of engineering in the UK has come into *Nature's* hands. After almost two years of preparation, the 65,000 word report contains 78 recommendations, the most important of which is the recommendation that an Engineering Authority be established with parliamentary powers to implement a restructuring of the engineering profession. The Authority would reduce the power of the universities over engineering education as it would have the power to withdraw accreditation from courses that do not meet its standards. Other features include a new three level hierarchy of engineering qualification and new names for engineering degrees.

Hailed as a solution to Britain's industrial decline, the report cites negative attitudes towards engineering and a lack of technological innovation as major factors that have kept Britain from trading "up market" in the international economy. A formal conference to initiate national discussion of the report's recommendations will be opened by the Secretary of State for Industry in London in mid-January. This will be followed up by wide circulation of the report to be coordinated by a Department of Education and Science steering committee. The steering committee will prepare working briefs which will be discussed at a second national conference late next year.

South Africa banned from IAEA meeting: South Africa was barred last week from the annual meeting of the International Atomic Energy Agency because of its apartheid policies. Forty nine non-aligned, socialist and developing nations out-voted 24 western nations on a resolution introduced by Nigeria, a leader in the effort to impose sanctions on the Pretoria government. The Nigerian delegation argued that South Africa should be banned because its apartheid policies meant that it did not represent the people of the country whereas the US delegate argued that banning South Africa would undermine attempts to prevent nuclear proliferation. It was the first time that any state has been barred from a meeting of the 22 year-old organisation.

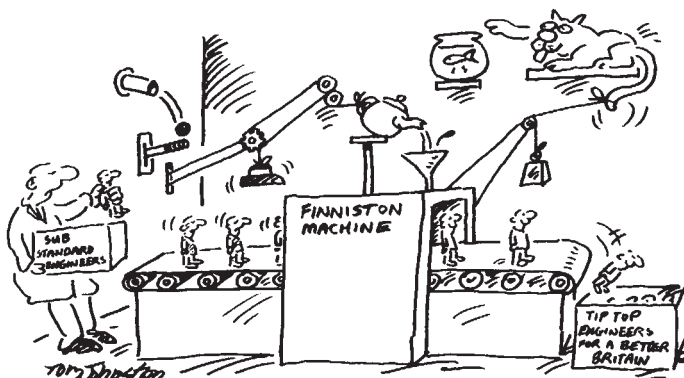
International conference planned for China: The Academia Sinica will be holding an international conference in Beijing next May on the Qinghai-Tibetan Plateau. The coverage will be multi-disciplinary, from geology to biological sciences. The aims of the meeting are to produce a comprehensive review of the results from scientific surveys conducted in the last three decades, and to enhance the international exchange of views and information. The Academia plans to organise immediately after the conference field trips to the plateau, in which participants including foreign delegates may take part. Prospective attendants from outside China may write in the first instance to the Foreign Affairs Bureau of the Academia Sinica.

Calls for inquiry into safety of 245T: The UK Shadow Minister of Agriculture, Mr Roy Mason has called for an inquiry into the safety and use of the herbicide 245T. He has joined several other individuals and organisations, including the National Union of Agricultural and Allied Workers and the Ecology Party, in expressing concern over the possible effect of the herbicide on animals and humans. At a meeting in London last week, Mr Tony Childs, a member of the Ecology Party told of a farmer in Somerset whose flock of sheep suffered 19 miscarriages after the Forestry Commission had sprayed woodland adjoining his fields with 245T. Some of the live lambs, he said, did not put on weight normally, began staggering and later died. Some second generation animals were also affected and all in all 90 beasts were lost. Mr Childs also cited the cases of two women who had both suffered miscarriages while their husbands were working as sprayers for the Forestry Commission. It was only several years later, as a result of increased publicity about the possible dangers of 245T, that the women had become suspicious that their miscarriages may have been linked to the chemical.

Regulations in the UK currently allow the use of 245T as a herbicide provided that its dioxin content does not exceed 0.1mg/kg.

Tactics behind UK nuclear decision leaked to Press: The UK government's planned expansion of nuclear power is to be kept as secret as possible to "avoid putting the government into a position of confrontation with the protestors". The government's tactics were discussed at a meeting of the Ministerial Committee on Economic Strategy attended by ten senior cabinet ministers on 25 October. The minutes of the meeting, marked 'Secret' and 'Confidential' were sent anonymously to the London magazine *Time Out* last week. The leak has stimulated the Prime Minister, Margaret Thatcher, to instigate an inquiry into how it happened. The UK is planning expansion of nuclear power which will involve the building of a demonstration pressurised water reactor, based on an American design, and at the same time keeping the option of building further advanced gas cooled reactors open.

Meanwhile the Central Electricity Generating Board with Government backing is resisting attempts by the Health and Safety Executive to obtain documents relating to the safety of the UK's American-designed pressurised water reactor.



Developing countries need more scientists

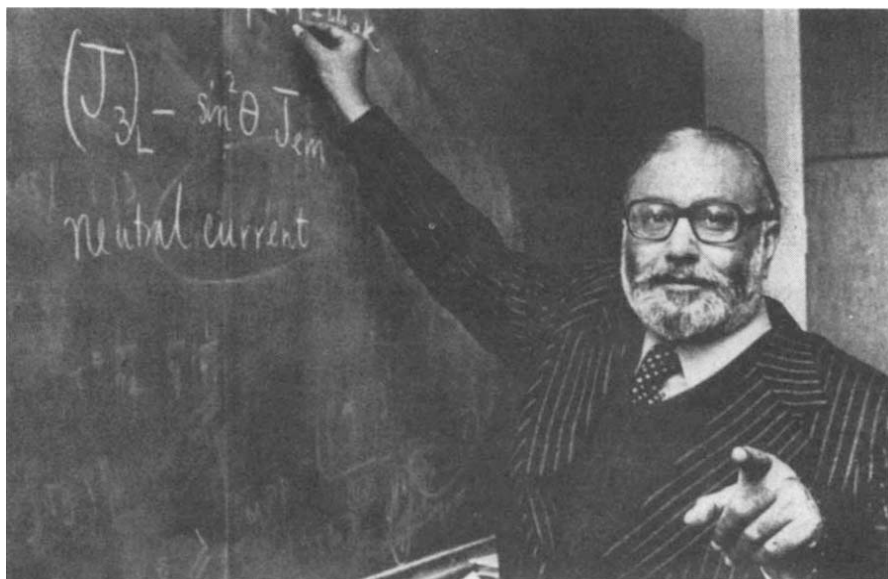
Western nations are not fulfilling their obligation to provide international centres of learning for scholars from developing nations. **Professor Abdus Salam**, co-winner of the 1979 Nobel prize in physics, makes a plea for new international universities of scientific teaching and research run by United Nations organisations.

THE history of science, like the history of all civilization, has gone through cycles.

Seven hundred and fifty years ago, an impoverished Scotsman left his native glens to travel south to Toledo in Spain. His name was Michael, and his quest to live and work at the Arab Universities of Toledo and Cordova, where the greatest of Jewish scholars, Moses Bin Maimoun, had taught a generation before. Michael reached Toledo in 1217 AD. Once in Toledo, Michael formed the ambitious project of introducing Aristotle to Latin Europe, translating, not from the original Greek, which he knew not, but from the Arabic translation then taught in Spain.

Toledo's school, representing as it did, the finest synthesis of Arabic, Greek, Latin and Hebrew scholarship, was one of the most memorable of international assays in scientific collaboration. To Toledo and Cordova came scholars not only from the rich countries of the East, like Syria, Egypt, Iran and Afghanistan — but also from developing lands of the West like Scotland. Then as now, there were obstacles to this international scientific concourse, because of economic and intellectual disparity between different parts of the world. Men like Michael the Scot and his contemporary, Alfred the Englishman, were singularities. They did not represent any flourishing school of research in their own countries. With all the best will in the world, their teachers at Toledo doubted the wisdom and value of training them for advanced scientific research. At least one of his masters counselled young Michael to go back to clipping sheep and to the weaving of woollen cloths.

Perhaps I can be more quantitative on this cycle of scientific disparity. George Sarton, in his monumental five-volume



Professor Abdus Salam demonstrating his Nobel prize-winning theory. He calls for a ten fold increase in scientists in developing countries.

History of Science chose to divide his story of achievement in science into ages, each age lasting half a century. With each half-century he associated one central figure. Thus 450 BC - 400 BC Sarton calls the Age of Plato; this is followed by half-centuries of Aristotle, of Euclid, of Archimedes and so on. From 600 AD to 650 AD is the Chinese half-century of Hsüan Tsang, from 650 to 700 AD that of I-Ching, and then from 750 AD to 1100 AD — 350 years continuously — we have the unbroken succession of the Ages of Jabir, Khwarizmi, Razi, Masudi, Wafa, Biruni and Omar Khayam; — Arabs, Turks, Afghans and Persians — men belonging to the culture of Islam.

After 1000 AD the first western names appear; Gerard of Cremona, Jacob Anatoli, Roger Bacon — but the honours are still shared with the names of Ibn-Rushd (Averroes), Moses Bin Maimoun, Tusi, and Ifn-Nafis — the man who anticipated Harvey's theory of circulation of blood. After 1350 AD, however, the developing world loses out except for the occasional flash of scientific work, like that of Ulugh Beg, the grandson of Tamurlane, in Samarkand in 1400 AD; or of Maharaja Jai Singh of Jaipur in 1720 AD — some 40 years after the setting up of Greenwich — who corrected the serious errors of the then western tables of eclipses of the sun and the moon by as much as six minutes of arc. As it was, Jai Singh's techniques were surpassed soon after, with the development of the telescope in Europe. As a contemporary chronicler wrote: "With him on the funeral pyre, expired also all Science in the East". And this brings us to this century when the cycle begun by Michael the Scot turns full circle, and it is we in the developing world who turn to the West for science. As Alkindi wrote 1100

years ago: "It is fitting then for us not to be ashamed to acknowledge truth and to assimilate it from whatever source it comes to us, even if it is brought to us by foreign peoples. For him who scales the truth there is nothing of higher value than truth itself; it never cheapens nor abases him."

During our present century, in the world of physics the first name is that of C.V. Raman — the Nobel Laureate of 1930, and there is then Yukawa, Tomonaga and Esaki; in between Lee, Yang and Ting. Most recently there is the great Jamaican economist, Sir Arthur Lewis, awarded this year's Nobel prize in economics.

In this context the question we must ponder is this; are we in the developing world today firmly on the road to a renaissance in sciences — as the West was in the 13th century at the time of Michael the Scot? Unfortunately the answer is No.

There are two prerequisites to this renaissance: one, the availability of places like Toledo for international concourse, where one can light a candle from a candle. Second, the passionate, consuming desire in our own developing societies to give the topmost priority to the acquisition of knowledge, and the removal of all internal barriers to this end. Unfortunately the prognosis here is not very bright.

And as to the first point, regretfully the opportunities for international scientific concourse are shrinking fast, with greater and greater restrictions in the traditional countries like the UK and US on the acceptance of overseas scholars, including those from developing countries. It is becoming increasingly clear that the developing world will need internationally run — UN and UNESCO run — institutions, universities of science, not just for research, but for the high level teaching of traditional technology and sciences, both

Professor Salam, Director of the International Centre for Theoretical Physics, and co-winner of the 1979 Nobel prize in physics with Sheldon Glashow and Steven Weinberg for the unified theory of the weak and electromagnetic interactions, first gave this address to the Unesco executive board shortly after the award of the Prize and the award by Unesco of the Einstein Medal.

pure and applied.

But for a series of accidents, Einstein may have been lost to physics, — such was the measure of financial, economic and other frustrations which he faced, even in a country like Switzerland. Unfortunately the same still applies so far as developing countries are concerned, in a far greater measure. Perhaps I can illustrate this with my own case.

The fact that I became and remained a research physicist is due to three accidents. First, the second World War: as soon as I showed some competence in science, my well-wishers, my parents, and all those around me, destined me for a career in the then prestigious Indian Civil Service. As it happened, with the war the civil service examination was suspended for the duration. But for this I would be a civil service functionary today. The second accident, which sent me to Cambridge for research, was again connected with the war. The then prime minister of my home State, the Panjab, collected some funds for the "War Effort". The war ended; the funds were left unutilized. He decided to institute "Small Farmers' Son's Scholarships" for study abroad. A number were offered; I was one of those fortunate to be selected, and sailed the same year — 1946 — to Cambridge. Several other scholarships were awarded, but unhappily the other scholars were only promised admission for subsequent years. In between, the subcontinent was partitioned and with it the scholarships disappeared. The entire exercise of the then prime minister succeeded in one thing only — in sending me for research at St John's College, Cambridge, where Professor Dirac lived and worked.

Centre for Theoretical Physics

The third accident happened after I returned to Pakistan, both to teach, and to try to found a school of research. In no uncertain terms, it was made plain to me that this was impossible. I must either leave physics research, or my country. With anguish in my heart, I made myself an exile — and it was this anguish which led me to propose the creation of an International Centre for Theoretical Physics, with the most active sponsorship of the government of Pakistan and other developing countries. The idea was to award what we call "Associateships" of the Centre, so that a deserving young man may spend his vacation in an invigorating environment in close touch with his peers in research, to charge his batteries with new ideas, while still spending nine months of his academic year in his own country, working at his university.

With UNESCO's active help, and with very generous assistance from the government of Italy and the town of Trieste, the Centre was created by the International Atomic Energy Agency (IAEA) in Trieste in 1964. UNESCO

joined as equal partners with IAEA in 1970. Over the 15 years that the Centre has existed now, it has veered from emphasis on fundamental and basic physics towards subjects on the interface of pure and applied physics — subjects like physics of materials, physics of energy, physics of fusion, physics of reactors, physics of solar and other unconventional sources, geophysics, physics of oceans, and deserts, systems analysis — this, in addition to high energy physics, quantum gravity, cosmology, atomic and nuclear physics and mathematics. This shift from pure to applied physics was not made because we thought that pure physics is less important for developing countries. It was simply that there was not any other international institute responsive to the needs of technological hunger involving the discipline of physics. Every year around 1,500 physicists — half from developing countries — spend of the order of six weeks or more at the Centre attending extended symposia or research workshops. The Centre has brought credit to UNESCO besides strengthening physics in the developed and developing world.

But over the 15 years that I have directed the Centre, I have felt more and more strangled, and never more so than now. I used to pride myself on spending half a day every day in research, half a day in administration. Progressively over the last five years this has become impossible. This is not because the task of administration has become more arduous; it is simply because the uncertainty of the Centre's standing in the ecology of international institutes has increased, despite its success, despite its demonstrated need. Its very existence is uncertain from year to year.

Briefly, half the budget of the Centre comes from the Italian government; the other half is shared between IAEA and UNESCO. I am fully aware of UNESCO's limitations in shouldering the responsibilities for such an enterprise; UNESCO defined its mandate 24 years ago as a catalyst of new institutions and not as their long-term sustainer. The realities of the situation now demand a revision of this mandate and newer stable funds to achieve this.

As I said earlier, the world needs today international institutions with requisite stability, for example, on the applied side, institutes like the Wheat and the Rice Research Institutes; and the educational and physics side, institutes like the International Centre for Theoretical Physics. To my distinguished colleagues on the Executive Board of UNESCO I say: that if you cannot find ways and means of keeping alive an initiative you took in 1970, and one now universally acclaimed and recognized to be essential to the health of physics in the developing world, then no one else can and no one else will. Institutes like the International Centre for Theoretical Physics must become part of the normal, continuing, stable United

Nations scene; otherwise the science and technology gap of the North and the South will never, never be bridged.

In science, as in other spheres, this world of ours is divided between the rich and the poor. The richer half — the industrial North and the centrally managed part of humanity — with an income of \$5 trillion, spends 2% of this — some \$100 billion — on non-military science and development research. The remaining half of mankind — the poorer South, with one fifth of this income (one trillion dollars) — spends no more than \$2 billions on science and technology. On the percentage norms of the richer countries, they should be spending ten times more — some \$20 billions. At the United Nations-run Vienna Conference on Science and Technology last August, the poorer nations pleaded for international funds to increase the \$2 billions to \$4 billions. They obtained promises, not of \$2 billions, not of \$1 billion, but only one seventh of this.

Three appeals

I would like to conclude with three appeals. The first to the international community — both of governments and of the scientists. A world so divided between the haves and the have-nots of science and technology cannot endure; at present an International Centre for Theoretical Physics (with a budget of \$1.5 millions) is all that is internationally available for physics in 100 developing countries. Compare this with European joint projects involving physics alone, of a half billion dollars annually.

My second appeal is to the developing countries. In the end, science and technology among them is their own responsibility. Speaking as one of them, let me say this: your men of science are a precious asset. Prize them, give them opportunities, responsibilities for scientific and technological development of their own countries. The goal must remain to increase their numbers tenfold, to increase the \$2 billions spent on science and technology to \$20 billions. Expenditure on science will pay tenfold.

And then finally, and in all humility, I wish to make a particular appeal to my brothers in the Islamic countries. To some of you Allah has given a bounty — an income of the order of \$60 billion. On the international norms these countries should be spending \$1 billion annually on science and technology. It is their forebears who were the torchbearers of international scientific research in the 8th, 9th, 10th and 11th centuries. Be generous once again. Spend the billion dollars on international science, even if others do not. Create a Talent Fund — available to all Islamic, Arab and developing countries, so that no potential high-level talented scientist is wasted. My humble personal contribution to this Fund will be all I possess — the \$60,000 the Swedish Academy has so generously awarded me. □

Science in Turkey: choosing the wrong priorities



Research geared to local problems could initiate a much needed boost to the Turkish economy. But Turkey's scientists and policy-makers are still intent on pursuing western science and the transfer of technology from the West writes **Ziauddin Sardar**.

LAST month, 55-year old former engineer, Suleman Demirel, formed a 'minority' government, composed entirely of his Justice Party, to lead Turkey out of its political and economic chaos. Demirel replaces Bulent Ecevit who resigned when his Republican Peoples Party suffered a crushing defeat in the November by-election to the National Assembly and the partial elections to the Senate. The other two major parties in Turkey — the Milli Salamat and the National Action Parties — promised to support the government, but declined to form a coalition.

Mr Demirel, now Prime Minister for the sixth time, asked the Turkish people for a "period of patience and confidence". He inherits a country torn apart by violence and on the verge of economic collapse, with inflation running at over 70% and unemployment approaching 25%. Unable to pay its short-term debt — \$2 billion of which is already overdue — Turkey has probably the worst short-term, debt-repayment problem yet to arise in world finance. Internal debts are increasing rapidly. Government-run industries — half of Turkey's industrial base — lost almost \$2 billion last year, a burden which must be met by the deficit-ridden treasury.

Turkey is struggling to modernise along western paths of development. It started along this course with the republican policies of Kemal Ataturk after the fall of the Ottoman Empire in 1923. Ataturk's modernistic reforms sought to transform Turkey almost over night into a western-style secular society. When he died in 1938 he left a legacy of ruthless westernisation that was followed brutally by his successors. Academic reforms began when the old *dearulfunun* were changed to universities with an accompanying change in the philosophy and method of research and education. Research in Turkey is still very much along western lines even though it has done little to solve the country's problems.

Turkey's current economic difficulties are having a major effect on universities and research institutes. The present situation at the Middle East Technical University (METU), in Ankara which has a high international reputation, is typical of the situation at other Turkish universities. "We are working under tremendous political and economic pressures", admits Okan Tarhan, the newly appointed Vice President of METU. "METU has received more than its fair share of unrest and violence and we have been paralysed much longer than any other university in Turkey. But we have made up for much of the lost time so that now we are only four months behind in our teaching schedules." METU has caught up with the lost teaching time by cutting its semesters from 18 to 16 weeks and by adding an extra semester to the academic year. But research time cannot be made up so easily.

"Our research projects have suffered most from the upheaval", explains Tarhan. "Many of the time-bound projects have had to be abandoned. Our post-graduate students are in particularly bad shape. Some of them have been waiting over three years to commence their experiments again."

Research has also come under heavy pressure from teaching. METU staff are too overburdened with teaching to find the time for research. "We are facing an acute shortage of staff", says Tarhan. "Faculty members are leaving rapidly to find jobs outside the university. Many are leaving Turkey altogether." On the other hand, student enrolment is increasing rapidly and the university is obliged, by law, to accept more and more students. "Currently, there are 12,000 students at METU," explains Tarhan. "There is an annual increase of 2,000 students per year. We are forced to accept these students, yet the university cannot cope with such a student population. Thus, our depleted teaching staff is continuously overloaded."

Reflecting the situation in the country as a whole, METU also faces an acute shortage of foreign exchange. "Because of this shortage", explains Tarhan, "we cannot buy chemicals and apparatus for our laboratories. Many of our postgraduate projects and much of our research is thus at a standstill. Some have been held up for as long as a year for nothing more than 5g of an everyday chemical, or because common equipment needs repair or has to be replaced. Our library is starved of vital journals and members of our academic staff are unable to attend international meetings".

The situation in the government-run research organisations is only slightly better. Government research in Turkey comes under the Scientific and Technical Research Council of Turkey (TUBITAK) which was established in July 1963. According to its General Secretary, Dr Tevfik Karabag, "TUBITAK assists the government in formulating the national policy to be pursued in fundamental and applied research in positive sciences." TUBITAK runs six research groups and the Marmara Scientific and Industrial Research Institute, one of the largest multidisciplinary research centres in Turkey.

According to Professor Haki Oranc, Assistant Secretary General for Technical Affairs at TUBITAK, the research groups are in dire need of staff, spare parts, chemicals and international help. "While a certain amount of theoretical research in mathematics and physics continues, experimental work is almost at a standstill." Worst hit by the economic stagnation are the Mathematical, Physical and Biological Sciences Research Group standstill. All TUBITAK research groups need to be revitalised with an injection of funds. Even a small amount of attention from UNDP and other international organisations would help to put Turkish research back on course".

This is particularly true of the Marmara Scientific and Industrial Research Institute (MSIRI) at Gebze. It was founded in 1972 after TUBITAK had carried out two extensive surveys which revealed that research in Turkey tends to be secondary to education and administration. The universities' research projects are of little relevance to the needs of Turkey and the report recommended the development of applied research in industry or at research institutes outside the universities.

Thus MSIRI was created to centralise industrial research and promote target-orientated research. It now has eight units whose interests range from operational research and applied mathematics to materials, nutrition and food and electronics. Its achievements are equally broad, from the prevention of corrosion by humidity control at the Bosphorus suspension bridge to better utilisation of tea by-products and wastes.

But despite the efforts of TUBITAK and the success of MSIRI, research activities in Turkey are neither coordinated nor directed towards the needs of the country. Turkish industrial enterprises do not

bother to improve the quality of their existing products or develop new ones; they operate on the basis of a straight transfer of technology, chosen randomly and haphazardly. Many institutions, such as the Electrical Power Resources and Survey Department and the Sugar Institute operate as isolated enclaves, repeating efforts and competing for resources.

Much of the research carried out at Turkish universities follows the patterns and priorities of research in western institutions. Pride of place is given to high energy and theoretical physics and astronomy, followed closely by pure chemistry and biology. Turkish scientists actively engaged in research are preoccupied with "basic research" in "pure science" seeking "an international reputation".

Metiu Bara, Assistant Dean of the Faculty of Science, University of Istanbul says "I am doing research on the action of gravity on plant metabolism, which is the field of my doctorate. I cannot honestly claim that I am doing anything for Turkey, but this is the tradition here. You do not become prominent by working on local

problems; in fact, that is a good way of losing respect amongst your colleagues. Prominent Turkish scientists will have nothing to do with local problems." That, in a nutshell, is the tragedy of the Turkish scientific community.

However, the stagnation of research and development in Turkey could well be a blessing in disguise, providing the Turkish scientific community with time to rethink their priorities and policies. Some changes are already taking place. The scarcity of funds, for example, is forcing university researchers to undertake consultancy work and contract research from industry. Thus at METU, the problems of pure physics and chemistry are now being replaced by such projects as the development of flotation methods for the transport of mined chromium, and the enlargement of the harbour for the Ereğli steel and iron factories. The government is also showing more interest in applied research projects. "Now most of our government backed research is dominated by immediate, practical problems", says Tarhan. Examples of such projects at METU are an examination of sewage and environmental

Earthquake research begins to be taken seriously

ON 24 November, 1976 an earthquake struck Caldiran, a sub-district of the eastern province of Van. The death toll was 3,840; 497 people were injured and 9,232 dwellings were either totally destroyed or damaged beyond repair. The Caldiran earthquake caused 415 deaths per 1,000 destroyed dwellings, the highest ratio recorded in Turkey for over ten years.

After a year's investigation, a joint team from the Earthquake Research Institute of the Ministry of Reconstruction and Resettlement and the Earthquake Engineering Research Institute of the Middle East Technical University, published a report strongly criticising government policy. It claimed that the Turkish government does not have a consistent policy on earthquakes, reacting only to large earthquakes, with hasty measures for emergency resettlement. Furthermore, the government has not given people living in the earthquake zones information on appropriate building materials.

But the report's major accusation is that earthquake risk is not taken seriously in the urbanised and industrial regions. Earthquake zones of the North Anatolian fault region cover 91% of Turkey, and 95% of the population lives in the danger area. In both town development and industrial location, however, the 'aseismic code', prepared by the Earthquake Research Institute, is not being implemented. Furthermore, seismic risk is not being considered as a factor in the selection of sites for

industrial and energy producing complexes.

According to Nejat Bayulke, chief engineer at the Earthquake Research Institute, the report produced significant changes in government policy. Since its publication, "work on earthquake zone mapping has been stepped up and a major campaign to inform the public about the hazards of earthquakes has been launched. Moreover, the earthquake design and construction code has been enforced more vigorously."

To assist in more accurate prediction, the institute is installing a country-wide

strong-motion accelerograph network, "We aim to install an average of 15 accelerographs per year within the seismically active regions", says Bayulke. In addition, it is carrying out microzoning investigations at large residential regions and industrial zones to minimise earthquake damage.

The institute has already installed some 80 accelerographs. But, as Bayulke points out, "these are by no means enough for an adequate network. We need 500 accelerographs. But where is the foreign exchange for us to buy the remaining 420?"

Caldiran after the earthquake: government criticised for ignoring seismic risk.



pollution in Konya, Isparta, and many studies on highway and urban traffic control.

A slight shift of emphasis is also detectable in the fourth Five Year Development Plan, presently being prepared by the State Planning Organisation. Although the new plan retains an emphasis on the transfer of technology and the pursuit of prestige science, it also acknowledges that Turkey must create new technologies of its own. Also, states the plan, "establishment of R & D units functioning with an organic link with industry (and) aiming at results directly related to industrial production, will be encouraged". To achieve this goal, the state will increase its expenditure on R & D. Turkish R & D expenditure has been increasing slowly since 1964. Between 1972 and 1977, it increased by 33.3% from 0.37% to 0.799% of the GNP — but it has still to cross the 1% barrier.

Unfortunately, the originality of the plan ceases at this point. It sees "technological progress" and "socio-

economic objectives of national economy" purely in terms of transferring and securing western technology. To support the adaptation and absorption of new technologies, says the plan, "scientific and technological research, surveys, engineering and other industrial studies will be encouraged and an effective cooperation among public institutions, scientific and educational bodies and private establishments will be secured".

Much of this transfer of technology will however take place in a haphazard manner, for there is no central organisation to coordinate technological activities in Turkey. Industry acts on its own initiative and the various scientific and research organisations make approaches to international organisations. Laws and regulations governing the transfer of technology form a complex maze, with several bodies — the Ministry of Finance, the Ministry of Health and Social Welfare as well as the State Planning Organisation — trying to control and direct the various activities of production, adaptation,

absorption and standardisation.

The plan recognises that the chief method of reducing unemployment is to promote labour intensive technologies — but these are considered largely to be obsolete and a hurdle to "technological progress". On international cooperation, the plan states "an effective and continuous cooperation especially with countries which are endowed with economic capabilities and wants which are similar to ours will be emphasised".

Turkey has had more than two decades of pursuing prestigious science and the transfer of technologies with the result that it has become dependent on the United States and Europe, a point brought to the fore by the US embargo after the Turkish invasion of Cyprus. The present economic crisis in Turkey is a direct result of these policies. It seems that Turkey is set to pursue, once again, the very science and technology policies that have produced the present economic crisis — dependency and unemployment and stagnation in scientific research. □

Turkey goes nuclear — with Sweden's help

In an abrupt about-face, Turkey has opted to rely on nuclear energy for the bulk of its electrical power by the year 2000. At present the country's energy requirements are met by five primary fuels: lignite, hard coal, oil, wood and dung. Half is produced by the domestic and industrial consumption of lignite, with the remainder being electrical power generated hydro-electrically (40%) and by coal- and oil-fired thermal power stations (10%).

Turkey's third Development Plan, in operation now, places emphasis on thermal power generation, including natural gas and geothermal resources, and on the further development of hydroelectric power with new plants on the Lower Euphrates. In mid-1978 Turkey was investing heavily in thermal and hydro-electric power with economic agreements with the Soviet Union and Czechoslovakia, and shortfalls in production being made good by energy exchanges with Bulgaria. Nuclear energy, seen within the Development Plan as contributing only 10% of Turkey's power, seemed a rather distant need. Only one nuclear power station was planned, a 600 MW plant to be completed in 1985.

But in the past few months, Turkey has come to see nuclear power as the only solution to an impending crisis. According to Dr Orhan Yesin, Deputy Secretary General of Technical Affairs in the Turkish Atomic Energy Commission, "Turkey's energy position is abysmal". Present indigenous fuels are becoming scarce, and the country's present economic state precludes future dependence on imported oil. The Atomic Energy Commission, founded in 1956, "has now established a small cadre of trained manpower", says Yesin. "We hope to expand our research

and training facilities to meet future demands for trained manpower." They will certainly be necessary for the success of the new plan, which envisages that nuclear energy will contribute some 75% of the country's power needs in 20 years' time.

Why the sudden change of policy? Dr A Kutukcuoglu, Director of the Department of the Nuclear Power Plants, Turkish Electricity Authority, echoes Dr Yesin: "Turkey's energy needs are growing rapidly and there is no viable alternative except nuclear energy to meet these needs". According to Kutukcuoglu, hydroelectric energy will reach its maximum capacity around 1985, and the existing thermal and lignite plants will not be able to meet the country's energy demands. "We have calculated that by the mid-1990s we will face a minimum deficit of 10,000 MW in our energy demands. We plan to meet this deficit with nuclear energy. Of course, as the demand for energy will increase, the deficit will increase too. We hope to be prepared for Turkey's energy demands beyond the year 2000", says Kutukcuoglu.

Turkey has signed an agreement with the Swedish firm ASEA-ATOM to build the first nuclear plant by 1987/88 at Akkuyu, 35 km from the town of Silifki, on the Mediterranean coast. The second will be operational by 1992 and the Turkish Electricity Authority is actively looking for an appropriate site. After that, eight more plants will be built in rapid succession so that a network of ten plants is operating by the year 2000. "We have not worked out the details beyond the second plant at this stage and, of course, the completion of ten plants by the year 2000 is not definite," explains Kutukcuoglu. "But we are trying our best to be prepared before the demand

arises."

In view of the present economic state and credit worthiness of Turkey, however, how can it finance the nuclear venture? "Turkey is a member of the International Atomic Energy Agency, which has provided considerable help and assistance to Turkey. It provides assistance which involves equipment and technical aid to the universities in Ankara and Istanbul. The IAEA has helped us not just in planning and safety measures but also in securing finances", says Yesin. Dr Kutukcuoglu is more open: "the venture is being financed by project-bound credit obtained through ASEA-ATOM." Each plant will cost Turkey an estimated \$1 billion. Some 20-30% of this cost will be met by the Turkish government, while the rest will be put up by ASEA-ATOM. At present, it is envisaged that all the ten power plants will be built on this basis.

There has been some opposition to Turkey's nuclear plans, but this has arisen only in Akkuyu and Silifki, near the first nuclear site. The Silifki anti-nuclear group have held several demonstrations and there have been a few questions in parliament. But on the whole, most people are unaware of Turkey's shift towards nuclear energy. And as Yesin admits, "public relations between the Turkish Electricity Authority and the Turkish Atomic Energy Commission are good". According to Kutukcuoglu, "public opposition to our plans have been minimal and the parliament has not raised any questions, although a serious, full-scale discussion has not taken place. The Fourth Development Plan is committed to nuclear power. As long as the government is behind us, we are going ahead with our plans as best and as rapidly as we can." □

news and views

Cytochalasin action

from Dennis Bray

CERTAIN chemicals have come to stand for particular areas of biological research. Think of fluorescein isothiocyanate; 2-deoxyglucose; bis-acrylamide; α -bungarotoxin, anti-mouse immunoglobulin. Substances such as these are hitched so firmly to a line of research that you could almost use their monthly sales figures to measure scientific fortunes. They can be important for practical reasons and are often essential to a new technique, but sometimes the link is of a more fundamental nature and arises because their site of action is so close to the heart of a biological phenomenon. This is well-illustrated by the cytochalasins — a group of secondary mould metabolites with a remarkable ability to paralyse the movements of vertebrate cells (see Tanenbaum (ed.) *Cytochalasins: Biochemical and Cell Biological Aspects*, North-Holland, 1978).

When the discovery came in the mid-1960s, that the cytochalasins had an effect on the migration and division of tissue cells there was, at first, little interest. In those days cell motility was an obscure subject — a few anchorites here and there watched amoebae crawl around or produced extracts of blood platelets but that was all. And then, within a few years, both the drug and the discipline took the centre of the stage. Everyone, from the most myopic graduate student to the most harried administrator knew that cells move, and that they do so by a sort of muscle contraction, and that this is blocked by a new drug called cytochalasin — or is it colchicine?

The history of how it happened has yet to be written, but a timely article in *Science* (171, 135; 1971) from a group at Stanford, led by Wessells, certainly had great influence. Touching most of the bases it surveyed the aspects of the new science of cell motility — locomotion, intracellular movements, shape changes — and linked them to an immature knowledge of non-muscle actomyosin systems. The keystone of this edifice was the effect of the drug cytochalasin which, it was suggested, acted on cell movements by direct action on actin.

The tidal wave that followed grew

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because everyone realised that movements were important to the phenomenon they were studying — whether it was secretion, phagocytosis, morphogenesis or transport. An international authority on the sex life of the red kidney bean found an actin band on a gel and filaments in a micrograph and new horizons opened before him. An hypothesis was born — curiously fashioned from sliding and interdigitating filaments it needed only one test to make it fact. And yes — cytochalasin causes *coitus interruptus*. Small wonder then that there was a reaction from the establishment. To those familiar with, say, the precise world of X-ray diffraction of myofibrils, the new science was a quagmire of half truths. And nowhere was this more true than in the connection between actin and cytochalasin. The evidence was indeed slim. Little more than that the most obvious morphological disorder in cells treated with the drug was in the microfilaments. Attempts to prove a closer relationship were disappointing. Muscle contraction, actin activation of myosin ATPase, the salt-induced polymerisation of pure actin, decoration of actin with heavy meromyosin — all these were essentially unaffected. Even more incriminating was the finding that the wonder drug of motility had other effects. It was very good at blocking glucose uptake into the cell — working quickly and at low concentrations.

The use of cytochalasin continued but without a licence: in many laboratories it became *chemica non grata*. Only a few talented researchers persisted with the conviction that it was of central importance. Several stratagems were developed to prove this point and one of them — that described here — has at last been successful. It began with the synthesis of a hydrogenated cytochalasin derivative that was free of the unwanted side-effects of membrane transport functions (Atlas & Lin *J. Cell Biol.* 76, 360; 1978) and the use of this bait to fish for the motility receptor site. A binding complex could be found in the erythrocyte membrane which contained spectrin, actin and band 4.1 (Lin & Lin *Proc. natn. Acad. Sci. U.S.A.* 76, 2345; 1979; see also *News and Views* 281, 426; 1979). The complex had the property that when added to a solution of monomeric actin it caused an explosive polymerisation — an intriguing property

when you consider microfilaments in the cell. When it was found that cytochalasin inhibited this polymerisation the writing was indeed on the wall (Grumet *et al. J. Cell Biol.* 83, 316a; 1979).

Everything that has been learnt about the actin polymerising complexes recently — and a great deal is about to be published — is encouraging. It, or its close relative can be isolated from other tissues such as brain or platelets (Flanagan, *Fed. Proc.* 38, 339a; 1979); the complex can utilise actin sequestered by profilin — a low molecular weight protein that is abundant in the cytoplasm (Lin *et al. J. Cell Biol.* 38, 317a; 1979); the different types of cytochalasin affect actin polymerisation with the same relative potency they show towards whole cells (Lin & Lin *op. cit.*). And at last the eagerly awaited nub of the complex has been identified, the crucial molecule on which cytochalasin acts was revealed as — after all — actin (Grumet *et al. op. cit.*). Or, to be more precise, it is the growing end of an actin filament. Within the complex, it is thought that a short oligomeric fragment is held in such a way that it can act as a nucleation point. A similar effect can be produced if pure actin is bound to polylysine (Brown & Spudich *J. Cell Biol.* 80 499, 1979) or stabilised by chemical cross-linking (Grumet *et al. op. cit.*). The induction of polymerisation by these fragments, curiously in contrast to salt-induced polymerisation, is blocked by cytochalasin; this drug moreover binds to filamentous actin with a stoichiometry that indicates binding at one end. It is similar, many will recall, to the action of colchicine on microtubules.

And so — thanks largely to the incisive experiments of Lin and his colleagues — cytochalasin has been made an honest drug. It acts on a small nucleating fragment of actin sequestered by a group of other proteins associated with the plasma membrane. The normal function of the nucleating fragment within the cell is, presumably, to cause actin subunits to assemble into microfilaments; the stimulus for this has yet to be identified and could well come from the other side of the membrane. Cytochalasin, therefore, forms a causal nexus between actin and the membrane and cellular movements, and acts — who can doubt it? — at the heart of cell motility. □

A surprising muddle in the optic nerve

from Julian Lewis

STUDENTS of the brain bewail its complexity. But how complex is it? Neurones can be classified into discrete categories according to their shape, location and connections; and though the number of such types is large — perhaps thousands — and the catalogue not yet complete, still the classification brings about a vast simplification. Neurones in the same category behave in similar ways, and make similar connections. But that is not to say that they make identical connections. The cells of a given category are spread over a range of neighbouring positions, and though they may all send their axons to the same target set of neurones, they do not send them all to precisely the same site within that target area, nor do they send them to sites distributed within it at random. It is usual rather to find an orderly map of one class of neurones onto another. The crucial feature of such a map is that it is continuous: neighbouring points of the one set project to neighbouring points of the other.

Since continuous neural maps are so commonplace in the brain, and seemingly so fundamental to its plan, one might hope to discover some simple and general mechanism of cell behaviour by which they are all set up. This hope has not been realised. Twenty years ago it appeared perhaps that Sperry might have solved the problem with his theory of neuronal specificity, whereby each cell was deemed to be chemically distinct, and to connect selectively by matching its own chemical label with that of a correspondingly distinctive target cell. Evidence came mainly from experiments on fish and amphibians. In the most famous of these, the optic nerve of a newt was cut and its eye turned upside down. The nerve regenerated, visual connections re-formed, and the animal saw the world again; but it saw the world upside down. Sperry's arguments were ingenious and persuasive. But subsequent studies, especially of connections between retina and tectum, have undermined his theory, at least in its extreme form. It seems almost certain that the arrangement of retino-tectal connections depends on interactions between one presynaptic fibre and another, and not simply on a matching of a presynaptic with a postsynaptic label. Such interactions might indeed be expected *a priori* to underlie the preservation of neighbourhood relationships which is the essence of a continuous map. But if Sperry's theory does not seem entirely true, neither does it seem entirely false. Continuity alone

is not enough to define, for example, the orientation of a map or the precise positions of its boundaries and landmarks, and chemical markers such as Sperry envisaged very probably play a part here.

As evidence has accumulated against the strict form of Sperry's theory, some absolutists have sought to reject it utterly, and enthrone in its place another all-explanatory principle. In particular, there has been a revival of the suggestion, discounted by Sperry, that orderly maps can be understood as a consequence of the rules of mechanical guidance of axon outgrowth (Holder & Martin *Symp. Soc. exp. Biol.* XXXII, 275; 1978). That is, the axons may arrive at their targets in an orderly array because they have made the entire journey in an orderly array.

This idea has seemed plausible for connections between eye and brain, since there is evidence in many species that the axons all along the optic nerve are indeed ordered retinotopically. One of the most extraordinary instances has been described recently by Scholes (*Nature* 278, 620; 1979): in a cichlid fish, the optic nerve has the structure of a ribbon pleated longitudinally, with the radial coordinate of position in the retina mapping across the breadth of the ribbon, and the circumferential coordinate mapping across its thickness. In monkeys and some other mammals, it has long been supposed from degeneration studies that there is again an ordering, though of a simpler sort (Polyak *The Vertebrate Visual System*, Chicago, 1957). In this issue of *Nature* (page 720), Horton, Greenwood and Hubel deal a blow to this belief, so far as adult cats are concerned at least. They inject a small spot of horseradish peroxidase into the lateral geniculate nucleus; the peroxidase is taken up by the optic axons terminating at the site of injection and is carried back to the retina, where it labels the cells from which those axons derive. The labelled cell bodies form a small tight cluster. Thus fibres projecting to closely neighbouring points in the lateral geniculate originate from closely neighbouring cells in the retina. That much is no surprise. But in a handful of neurones the peroxidase labelling is so intense that the axons themselves can be followed in their courses; and it turns out that these axons, far from remaining neighbours throughout the optic nerve, diverge as they leave the retina and become dispersed among axons from other sites, only to converge to become neighbours again in the lateral geniculate. Electrophysiological tests corroborate this finding.

If there is order in the cat's optic nerve, it must be order of a very bizarre sort at best;

though the example of the cichlid fish might make us hesitate to conclude that there is no order at all, but only a random interweaving of axons. It is nevertheless hard now to believe that the continuous map from the cat's retina to its lateral geniculate could be the outcome of neighbourhood relationships maintained throughout the optic nerve.

The findings of Horton, Greenwood and Hubel may be construed by some as support for Sperry's ideas. But neuronal specificity is not the only device by which an orderly somatotopic map may develop despite disorder of the fibres *en route* from source to target. There are ways to establish continuity in a projection without having the neurones chemically individuated or their axons guided in parallel paths of outgrowth. In particular, electrical activity might play a part. Neighbouring cells in general will tend to be correlated in their time of firing. Thus a continuous projection can be set up if axons which fire at the same time tend to make synapses on the same or on closely adjacent targets (Willshaw & von der Malsburg *Proc. Roy. Soc. B* 194, 431; 1976). There is evidence for such a principle in the development of binocular neural connections in mammals and in amphibians (Keating *Phil. Trans. Roy. Soc. B* 278, 277; 1977); and a similar idea has been invoked to explain associative learning.

But the history of the problem of neural maps teaches caution. The theories are many and seductive, and it is dangerous to yield entirely to the charms of one alone. Each may represent only a part of the truth. Different vertebrate species, and different neural subsystems, may make their maps differently. □

Reinnervation of adult muscle

from Clarke R. Slater

THE neural control of muscle activity in vertebrates is based on a simple pattern of cellular connections. The highly branched axon of each motor nerve cell provides the exclusive innervation for each of a large number of muscle fibres. The simplicity and apparent stability of this pattern conceals considerable potential for adaptive change which is revealed when death or injury of motor neurones leaves some muscle fibres denervated. For then sprouts grow from the specialised terminals of the intact motor axons and reinnervate many of the denervated muscle fibres, often precisely at the site of the original nerve-muscle junction. This ability to compensate in the periphery for damage to motor neurones in the spinal cord is one of great practical significance not only in disease but also during ageing, for most of us will lose more than half our motor-neurones by the time we die. Although the nature of the signals which

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control the effective reinnervation of adult muscle has remained a mystery for many years, several recent studies have begun to shed light on this important aspect of neuronal plasticity.

Many explanations have been suggested for the initiation of axonal sprouting. One which has received some experimental support is that sprouting from intact axons is triggered by the chemical breakdown products of other axons degenerating nearby. But this is clearly not the whole story for substances such as botulinum toxin, which prevent the neural activation of muscle, induce sprouting without causing any obvious axonal degeneration. A few years ago, Brown and his colleagues found that when mouse muscles poisoned with botulinum toxin were kept active by direct electrical stimulation, sprouting was greatly reduced (*J. Physiol.* 267, 42P; 1977). They now present further evidence which supports their view that in a partially denervated muscle one, though not the only, source of the stimulus for axonal sprouting is the denervated muscle fibres themselves (this issue of *Nature*, page 724).

They have found that when a muscle is partially denervated, by cutting some of its motor axons, direct electrical stimulation of all the muscle fibres largely prevents sprouting from the intact axons. On the other hand, if the nerve itself is stimulated, so that only the innervated fibres are active, then sprouting occurs normally. The possibility that the intense currents needed for direct muscle stimulation might inhibit axonal growth has been ruled out by showing that when all the axons are damaged by crushing the nerve, their regeneration is not impaired by direct stimulation. Taken together, these observations suggest that the inactivity of the denervated muscle fibres is a key factor in the initiation of sprouting from intact motor axon terminals. Since those terminals are often not in direct contact with denervated muscle fibres, the authors point out that the sprouting stimulus must be able to spread some way within the muscle.

It has been known for many years that denervation causes important changes in muscle cells, making them susceptible to innervation by sprouts of growing motor axons and sensitive to the transmitter which those sprouts release. Recently it has become clear that inactivity of the muscle is an important factor in bringing about those changes. In this context the induction of sprouting from intact motor axons can be seen as a part of a concerted response to muscle inactivity, seemingly aimed at restoring functional innervation.

Once axonal sprouting starts, where does it stop? Many experiments have shown that in spite of the ability of denervated muscles to accept innervation at unusual sites, reinnervation is most

likely to occur at the site of the previous neuromuscular junction, where the axons stop growing and differentiate into mature presynaptic terminals. In a series of striking experiments, McMahan and his colleagues have shown in frogs that the regenerating axons seem to recognise the site of the neuromuscular junction by virtue of a specialised region of the extracellular basal lamina (*J. cell. Biol.* 78, 176; 1978). This structure, which consists largely of a special form of collagen, surrounds each muscle fibre and each motor axon (together with its sheath of Schwann cells) and is interposed between them at the neuromuscular junction. Although the function of the basal lamina is poorly understood, it is clear that when muscle fibres or axons are damaged and degenerate the basal lamina often persists to provide a 'scaffold' for their regeneration.

By damaging a muscle and preventing its regeneration, McMahan *et al.* have produced a situation in which axons, injured by crushing, grow back to an empty tube of basal lamina. Remarkably, the axon terminals end up in contact with exactly those regions of the basal lamina which were at the original neuromuscular junction (these can be identified by a number of structural criteria) and go on to elaborate several of the characteristic features of presynaptic terminals, even though no muscle fibre is present.

In these experiments, the precision with which the region of the vacated neuromuscular junction is reinnervated may owe much to mechanical constraints on the regenerating axon provided by the tube of basal lamina which surrounds it. But other observations point to the possibility of some more specific local clues at the junction itself. Thus even in the absence of a basal lamina tube — for example when the sprouts arise from existing terminals (see above) or when the nerve regenerates after being sectioned — reinnervation still occurs preferentially at junctional sites.

Bixby and Van Essen have found that even when 'foreign' axons are caused to grow into the region of the neuromuscular junctions in normally innervated rat soleus muscles, they occasionally come to innervate muscle fibres, apparently by displacing the soleus axons from the junctions (this issue of *Nature*, page 726). The 'capture' of these muscle fibres occurs only when the invading axons reach the junctional region, supporting the view that only there is the surface of a normally innervated muscle fibre specialised to enter into a functional relationship with a motor axon. These experiments also demonstrate, perhaps more dramatically than any before, that the association between a mature motor axon and the muscle fibres it innervates is a dynamic one capable of major change in the face of an external challenge, even if that challenge comes from axons that would normally

innervate quite a different muscle.

As yet, molecular labels which might enable a growing motor axon to recognise the junctional region of a muscle fibre have not been identified though one intriguing precedent exists. Acetylcholinesterase, the enzyme that breaks down the chemical transmitter soon after its release from the motor axon terminal, is firmly bound to the basal lamina at the junction, and persists after degeneration of the axon, the muscle fibre, or both. What is important about the studies I have discussed is that they are beginning to identify those cells and cellular components that are the likely sources of the signals which control the effective reinnervation of adult muscle. This information is clearly essential for future studies of the molecular basis of those signals. □

New ways of measuring cadmium in man

from Wynford D. Morgan

CADMIUM is a ubiquitous metal, having widespread and increasing uses in various products including nickel-cadmium batteries, solar cells and electroplating. The element is not essential to man, being virtually absent at birth but gradually increasing with age by absorption from the diet, from smoking, and in some cases from occupational exposure. Although effects on health from acute exposure have now been largely eliminated as a result of better working conditions, nevertheless there is still concern over the longer term effects, on the kidney in particular, of prolonged exposure either at work or in unusual environmental conditions. Here the relatively new technique of *in vivo* neutron activation analysis (IVNAA) is of value in allowing measurement of the amounts of cadmium deposited in the organs of living subjects, and thereby providing the important middle step in the exposure-uptake-response sequence of events.

Cadmium is recovered as a by-product from the smelting and refining of zinc ores. Concentrations in air normally range from 0.001 to 0.05 $\mu\text{g m}^{-3}$ but may be as high as 5 $\mu\text{g m}^{-3}$ near a point source of emission such as a smelter. Within the workplace the current British Threshold Limit Value (TLV) is 50 $\mu\text{g m}^{-3}$, a figure which some people consider still to be too high. Seawater contains about 0.1 $\mu\text{g l}^{-1}$, non-polluted freshwater generally less than 1 $\mu\text{g l}^{-1}$, while the WHO limit for drinking water is 10 $\mu\text{g l}^{-1}$.

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Soil concentrations are usually less than $1 \mu\text{g g}^{-1}$ (1 p.p.m.) but may rise to approximately $100 \mu\text{g g}^{-1}$ as a result of aerial deposition, irrigation with contaminated water, or the application of sewage sludge-based fertilisers. In exceptional circumstances, as in the Somerset village of Shiphams, soil concentrations of up to 1,000 p.p.m. have been found. However, the uptake of cadmium in plants is not necessarily related to its concentration in the soil, as the bioavailability of the metal depends among other things on the soil pH.

For the general population, the main source of cadmium for non-smokers is food, the average diet containing 0.01 to $0.04 \mu\text{g g}^{-1}$. Depending on the quantity of food consumed, the daily intake in non-polluted areas is therefore in the range 10–80 μg per day compared with the recommended WHO limit of 400–500 μg per week. Only 5% may be actually absorbed in the digestive tract, and this again may be modified by the relative amounts of other elements such as calcium and iron, so the daily absorption through the gut is in the range 0.5 to 4 μg . Of equal importance is the fact that cigarettes contain 1–2 μg cadmium, of which 5% may be absorbed, so that a smoker of 30 cigarettes each day for 10 years may accumulate up to 10 mg of the metal in addition to a dietary intake of up to 15 mg in the same period.

What threat, if any, do such quantities of cadmium pose to human health? The metal accumulates gradually throughout life to a level of 10–60 mg at the age of 50, with approximately one half of the total being deposited in the liver and kidneys combined. Excretion takes place very slowly, the biological half-life of cadmium in these tissues being of the order of many years.

It is difficult to define a 'normal' value for cadmium, because even relatively small amounts have been implicated in a variety of diseases including high blood pressure. Nevertheless, concentrations of 15–100 p.p.m. wet weight in the kidney cortex (1.5 to 10 mg total kidney) and 1–10 p.p.m. (1.7 to 17 mg) in the liver would seem not to cause organ damage such as might result in proteinuria and disturbances in bone metabolism. After exposures to high levels of cadmium, the liver can contain 100 p.p.m. and the cortex 150 to 450 p.p.m. in the absence of renal dysfunction. Above this level, kidney damage can occur, resulting in proteinuria and loss of Cd from the kidney.

However, better information is required on the critical concentration of cadmium in the kidney cortex before damage does occur, and greater knowledge is needed of dose-response relationships in general. Unfortunately, existing methods of measuring blood and urine cadmium are inadequate in predicting body burden, while the more recent assays of urinary β_2 -microglobulin activity indicate the point

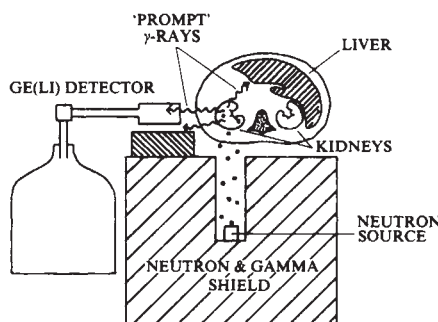


Fig. 1 Schematic illustration of the *in vivo* measurement of cadmium in the kidney.

at which damage has already occurred. The newer technique of IVNAA is therefore of value in providing non-invasive measurements of organ cadmium contents.

In essence, the method involves irradiating the organ of interest with a collimated neutron beam and counting on-line the prompt 559 keV gamma rays emitted during neutron capture in cadmium (see Fig. 1). The technique was pioneered by J. Fremlin's group at Birmingham University using the cyclotron as a neutron source (*Nature new Biol.* **236**, 182; 1972; *J. radioanalyt. Chem.* **19**, 207; 1974); but with the realisation that adequate neutron fluxes could be provided by isotopic neutron sources, portable instruments have since been constructed in at least five laboratories in Birmingham, New York, Swansea, Toronto and Baghdad.

The Birmingham device originally used a 10 Ci source of $^{238}\text{Pu}-\text{Be}$ with which a liver Cd concentration of 20 p.p.m. (2 s.d. of the background) could be measured for a radiation dose of 70 mrem (*Phys. Med. Biol.* **24**, 432; 1979). However, an improved version has been used in a survey of several hundred occupationally-exposed workers (*Lancet* **i**, 221; 1979) where evidence was obtained for the critical concentration in the kidney cortex being in the range of 200–250 p.p.m. Similar measurements of 21 Shiphams residents (*Lancet* **i**, 551; 1979) revealed smaller cadmium burdens but these were nevertheless much higher than would be expected in a non industrially-exposed group.

More sensitive instruments have been built at Brookhaven Laboratory, New York (*Phys. Med. Biol.* **22**, 1085; 1977) and in a joint University-Hospital project in Swansea, Wales (IAEA-SM-227/9, 1979). The latter uses a ^{252}Cf neutron source and achieves a detection limit in the kidney of 3.2 mg for an organ dose of 300 mrem. In a study by Cummins of 30 hypertensive subjects the mean kidney Cd content was not significantly different from 30 controls, matched for age, sex and smoking habit. In the United States Ellis (*Science* **205**, 323; 1979) has measured the additional body burden derived from cigarette smoking, while the same group (S. H. Cohn) has recently completed a

study of occupational exposure, using a system mounted in a mobile trailer (IAEA-SM-227/78, 1979).

Further instrumental improvements are possible by the use of better detector systems, while the availability of a pulsed neutron source of optimum energy would provide many of the advantages of Ettinger's original Birmingham system (*Kernteknik* **17**, 89; 1975).

Use of the technique has not yet changed current thinking concerning Cd metabolism in man, but as data accumulate, particularly from the important longitudinal studies on both 'exposed' and 'normal' populations we may be able to state more confidently whether current exposure levels are likely to be harmful either now or in the future.

Dihydrofolate reductase: the current story

from J. E. Gready

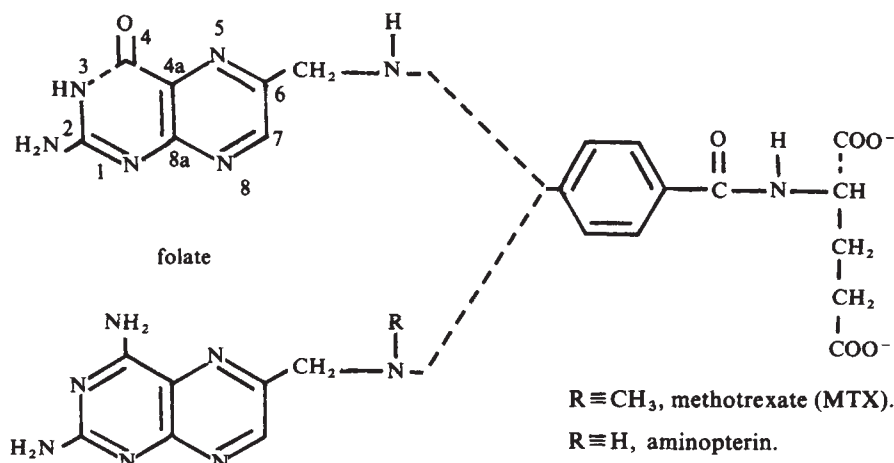
THE antifolate drugs methotrexate and aminopterin have been used in cancer chemotherapy, especially of leukaemia, for more than 30 years and their target in the cell is probably the best characterised of all the antitumour drugs. They inhibit the enzyme dihydrofolate reductase, competing with its natural substrates, and so block the production of tetrahydrofolate, an essential cofactor for DNA synthesis.

Because of its importance as a target not only for antitumour drugs but also for antibacterial agents such as trimethoprim and antimalarials such as pyrimethamine, the structure of dihydrofolate reductase (DHFR) and its interactions with its natural substrates (folate and dihydrofolate) and the inhibitors have been much studied, in the hope of finding useful clues to the development of novel inhibitory drugs. New results on the stereochemistry of the binding of dihydrofolate (DHF) and folate to DHFR, and also of their enzyme reactions with the coenzyme NADPH, however, have necessitated the revision of some current ideas, and in some ways pose more problems than they solve.

One of the most taxing problems in considering the action of the inhibitors is why methotrexate (MTX) — and similarly aminopterin — binds 10,000–50,000 times more tightly to DHFRs than does folate.

One difference is that bound methotrexate is protonated — almost certainly on N(1) of the pteridine ring system (see figure) — whereas the natural substrates are not. However, Hood and

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Roberts (*Biochem. J.* 171, 357; 1978) showed that only about one-third of the difference in binding energy between folate and methotrexate could be attributed to protonation, which accords with several spectroscopic studies indicating that as well as the protonation difference the two substrates are bound somewhat differently. Now new evidence is available (Charlton *et al. Chem. Commun.* 922; 1979) to show that the pteridine ring of the substrates is bound upside down compared with the inhibitor MTX.

Previous X-ray crystallographic studies of the binding of methotrexate to bacterial DHFRs (Matthews *et al. Science* 197, 452; 1977; *J. biol. Chem.* 253, 6946; 1978) had provided a picture of the pteridine ring bound in a hydrophobic pocket on the enzyme with the carboxylate side-chain of an aspartate residue (Asp-29) in close proximity to N(1) of MTX. Protonation of N(1) would allow formation of an electrostatic bond with an ionised carboxylate group ($-\text{COO}^-$). X-ray studies of the enzyme-MTX-NADPH ternary complex showed that NADPH was bound in such a way that a hydride transfer to C(6) would be physically possible. C(6) is the site at which reduction of the natural substrate dihydrofolate occurs. Furthermore the hydride would come from the A side of NADPH in accord with experimental results for the stereospecificity of the enzymic reduction. Taking into account that many of the enzyme interactions with MTX were with strongly conserved residues, the X-ray results in general pointed to a strong correspondence between the binding of MTX and that of the substrates and various deductions as to the natural reduction mechanisms were made.

The new results however, upset this picture considerably. They show that the pyrazine rings of both folate and DHF are reduced on the opposite face to that predicted from the X-ray structure for the inhibitor complexes. Thus the pteridine ring of the substrates is bound to the enzyme upside down compared with the corresponding ring of MTX. The exact nature of this inversion — for example,

inversion about which axis of the pteridine ring, and whether the binding of the phenyl moiety of MTX is preserved, is as yet unknown. It should be noted that B.R. Baker put forward the possibility of an inverted pteridine ring more than 10 years ago.

New amino acid sequence data for more DHFRs have also thrown doubt on the assessment of the importance of the interaction of the protonated N(1) of MTX with the Asp-29 residue. For now that the sequences of three mammalian enzymes are available it is clear that Asp-29 is only conserved in some enzymes — so far bacterial only — being replaced by asparagine or glutamic acid (depending on the method of aligning the sequences) in mammals. Although glutamic acid could possibly interact with the protonated N(1) of MTX in the same way as aspartic acid, the different binding of the substrates with respect to both protonation and inversion of the pteridine ring makes the explanation of the natural function of these acidic residues — located in a primarily hydrophobic pocket — even more perplexing.

When Charlton *et al.*'s results are applied to the question of the reduction mechanism the most reasonable assumption is that folate is initially reduced at C(7) and then at C(6), while DHF is reduced at C(6). In general the enzymic reduction of folate proceeds at a much lower rate than that of DHF. By analogy with other dehydrogenases it has been generally assumed that pre-protonation of the pteridine ring — specifically at N(8) for the initial reduction step in folate and N(5) for reduction of DHF — would facilitate the enzymic process. However there is no evidence for protonation of either folate or DHF in the binary complexes. Considerable attention has been given to possible proton donors on the enzyme. Again by analogy with other dehydrogenases, the earliest theory implicated a histidine residue: however, it is now clear that there are no fully conserved His residues in DHFRs and the X-ray studies also fail to show a His residue anywhere near the pteridine ring binding

pocket. Later suggestions based on the X-ray studies implicated either Asp-29 or Thr-136 (as yet, fully conserved): although the precise placement of these residues with respect to the 'inverted' pteridine ring of the natural substrates is not known it is unlikely that direct proton transfer to either N(5) or N(8) would be possible, and almost certainly not to both.

It is obvious that DHFR is somewhat unusual in catalysing reaction at two substrate sites, even though they are adjacent. During enzyme evolution structural constraints at the active site may have precluded development of a residue side-chain capable of directly protonating both N(5) and N(8). An alternative mechanism for facilitating reduction may thus have evolved. Another point to emphasise is that there are two halves to this redox reaction — the substrate and the coenzyme reactions: specific activation of NADPH in this reaction has not been formally discussed in the literature. On general grounds it might be thought that some activation of coenzyme during binding to all dehydrogenases is likely.

The implication is that sufficient flexibility must be involved in coenzyme binding in the ternary complexes to allow direct hydride transfer to both C(6) and C(7). Also, although general considerations of the relative degrees of aromaticity of the respective pyrazine rings may provide a simple chemical explanation of the faster absolute rate of enzymic DHF reduction compared with that of folate, the large enzyme-species dependence for the relative rates argues for some specific contribution from enzyme binding. □



100 years ago

CAMBRIDGE UNIVERSITY

The stipends of the Professors, it is suggested, should be raised from their present level to correspond with the following scheme:—

Professors.

	£		£
Regius of Law.....	600	Experimental Physics..	750
Whewell.....	500	Mechanism.....	400
Regius of Medicine....	400	Astronomical Physics..	500
Anatomy.....	300	Woodwardian.....	500
Pathology.....	600	Botany.....	300
Greek.....	750	Mineralogy.....	300
Latin.....	750	Zoology and Compara-	
Arabic.....	500	tive Anatomy.....	600
Sanskrit.....	500	Physiology.....	600
Anglo-Saxon.....	500	Modern History.....	400
Lucasian.....	750	Thirlwall. Dixie.....	
Plumian.....	750	Knightbridge.....	400
Lowndean.....	600	Political Economy.....	300
Sadlerian.....	600	Mental Philosophy	
Jacksonian.....	600	and Logic.....	400
Chemistry.....	750	Music.....	200

From *Nature* 21, 11 Dec., 126; 1879.

Thrombosis and haemostasis

from Robert W. Colman, Gwen Stewart, Andre Budzynski, T. Roy Ittyerah, Stephanie Olexa, Edward Kirby, Miriam Fukami and Jeanette Piperno.

FOR over a century investigators have been fascinated by the mechanisms by which blood remains in a fluid state while circulating *in vivo* but forms a fibrin-platelet thrombus *in vitro* or in disease states. Current research directed at solving this problem was presented at the VIIth International Congress on Thrombosis and Hemostasis held in London earlier this year*. The presentations were notable for the wide range of approaches and disciplines applied to the study of haemorrhage and thrombosis. The hypothesis was presented by J. Vane and S. Moncada (Wellcome Research Laboratories, Beckenham, UK) that PGI₂ (prostacyclin) synthesised by endothelium cells lining vessels is responsible for preventing thrombosis in the normal vessel. This prostaglandin derivative increases platelet intracellular cyclic AMP levels, thus inhibiting platelet aggregation. This attractive concept was criticised by several groups at the meeting. J.B. Smith and his colleagues (Jefferson Medical College, Philadelphia) found that an antibody which could neutralise the platelet inhibitory action of PGI₂ failed to alter the *in vivo* bleeding time. Moreover, G. deGaetano and his colleague (Istituto di Ricerche Farmacologiche, Milan) demonstrated that PGI₂ failed to protect rats against venous thrombosis. Thus, PGI₂ may represent one but not the only endogenous substance responsible for the nonthrombogenicity of the blood vessel wall.

Haemophilia is one of the most common congenital haemorrhagic disorders. Yet the protein which is missing or abnormal, anti-haemophilic factor, has never been purified to a homogenous state. For this reason assays for detection of carriers for prenatal diagnosis in fetal blood have been limited to coagulant assays or immunoassays of the related protein, von Willebrand's factor. L. Hoyer and his group (University of Connecticut Health Center, Farmington) reported on the use of an immunoradiometric assay based on human inhibitors but these naturally occurring antibodies are limited in availability. G.A. Vehar and E.W. Davie, (University of Washington, Seattle) however, reported a 500,000-fold purification of coagulant factor VIII which may pave the way to simpler assays based on heterologous antibodies.

The role of phospholipid micelles in blood coagulation has been appreciated for many years but the anatomical localisation in the organism has not been clear. R.F.A. Zwaal and his associates (States University of Limburg, Maastricht) presented data

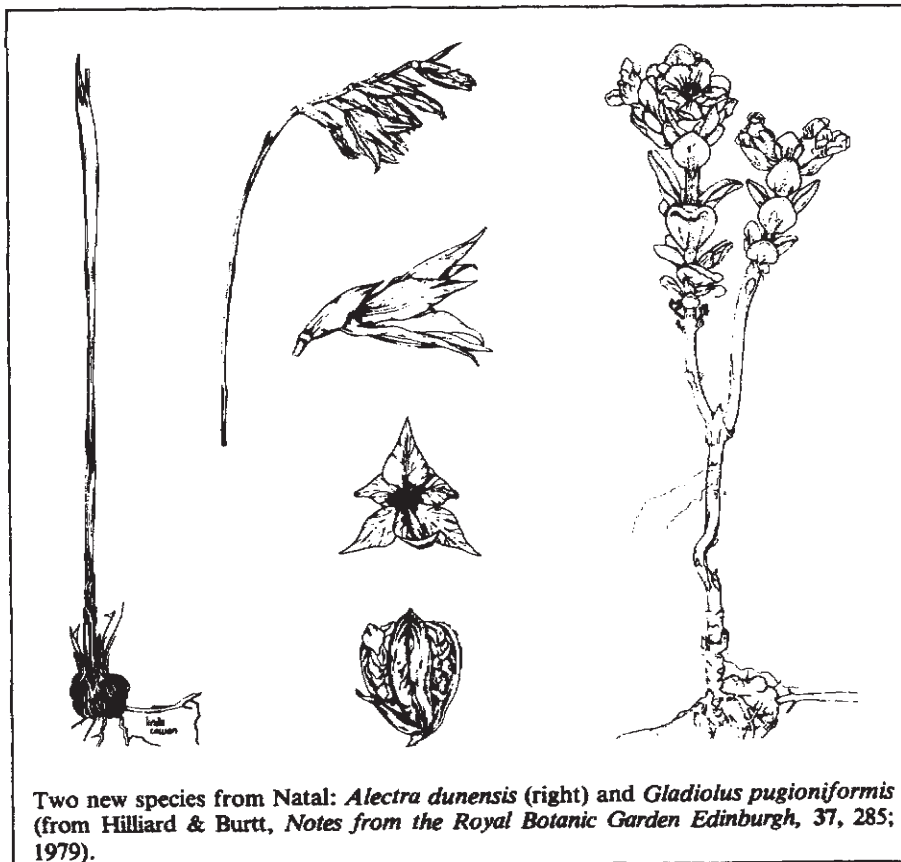
indicating that the phospholipids may be on the internal plasma membrane of the platelet and exposure may occur by translocation of these phospholipids to the external platelet membrane. Further reactions at the platelet surface involve the binding of an accelerator of blood coagulation activated factor V (K. Mann *et al.* Mayo Clinic Foundation, Rochester) followed by the attachment of the serine protease factor Xa which then converts prothrombin to thrombin (J. Miletich & P. Majerus, Washington University, St Louis). The source of the factor V may be the alpha granules of platelets according to T. R. Ittyerah, R. Rawala and R. Colman (Temple University, Philadelphia). These investigators found that bovine platelet factor V is a single chain of molecular weight 270,000 which is released by collagen from platelets and in the process is proteolytically converted to an activated form. Thus, the entire middle phase of blood coagulation occurs on the platelet surface and requires activation of the platelet. P. Walsh (Temple University, Philadelphia) and J. Griffin (Scripps Clinic, La Jolla) reported that platelets may accelerate the early contact phase of blood coagulation, further emphasising the functional interdependence of coagulation and platelet activation. This theme was further emphasised by the findings of D.

*15-20 July, 1979.

Phillips *et al.* (St Jude Children's Research Hospital, Memphis). These investigators found that thrombin aggregates platelets forming a filamentous complex which contains glycoproteins II_b and III of the platelet membrane. Yet another example of the critical interactions between coagulant proteins and platelets is the well known requirement of fibrinogen for platelet aggregation by ADP. J.S. Bennett (University of Pennsylvania) T.S. Edgington *et al.* (Scripps Clinic, La Jolla) and M. Zucker *et al.* (New York University Medical Center) have all demonstrated that ADP stimulation of platelets makes available fibrinogen binding sites on the platelet sticking one to another in the fibrin thrombus.

The fibrin thrombus once formed will occlude the vessel until lysed by the enzyme plasmin. Collen (University of Leuven) presented an integrated theory explaining lysis of the clot in the gel phase but not fibrinogen in the fluid state. Fibrin binds both plasminogen activators and plasminogen. Plasmin formed remains bound and is protected from the potent, naturally occurring proteolytic inhibitor α_2 -antiplasmin. Determination of components of the fibrinolytic system may thus allow better control of thrombolytic therapy.

As a whole, the meeting emphasised that to elucidate the mechanisms of haemostasis and the pathophysiology of thrombosis, a knowledge of cell biology, biochemistry and immunology must be applied to study vessel wall cells, plasma coagulation factors and platelets. □



Two new species from Natal: *Alectra dunensis* (right) and *Gladiolus pugioniformis* (from Hilliard & Burt, Notes from the Royal Botanic Garden Edinburgh, 37, 285; 1979).

The authors are at the Specialised Center of Thrombosis Research, Temple University School of Medicine, Philadelphia.

articles

Particulate organic matter flux and planktonic new production in the deep ocean

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Primary production in the oceans results from allochthonous nutrient inputs to the euphotic zone (new production) and from nutrient recycling in the surface waters (regenerated production). Global new production is of the order of $3.4\text{--}4.7 \times 10^9$ tons of carbon per year and approximates the sinking flux of particulate organic matter to the deep ocean.

THE sinking flux of particulate organic carbon (POC) to the deep sea has been estimated from a geochemical, two-layer box model of the global ocean to be 10% of the surface ocean production¹. If surface production is in the range $20\text{--}45 \times 10^9$ tons C yr⁻¹ (ref. 2), then the sinking flux would be about $2.0\text{--}4.5 \times 10^9$ tons C yr⁻¹. We estimate the sinking flux of POC in the deep ocean by assuming that new production, as defined by Dugdale and Goering³, is quantitatively equivalent to the organic matter that can be exported from the total production in the euphotic zone without the production system running down. Regenerated production constitutes the remaining production and is based on nutrients recycled through the food web of the surface ocean. The principal source of nutrients for new production is the upwelling and diffusion of nutrients from deeper water into the euphotic zone. Secondary sources are terrestrial (rivers, runoff, sewage), atmospheric (nitrate in rain, for example), and nitrogen fixation. Export fates of new production, other than sinking (and with a lower flux rate)⁴, include the fish and shellfish catch, deposition of guano on land by marine birds and the like.

Nature of the sinking POC

All the ocean contains sinking particulate matter which is the source of food for the microorganisms and animals of the deep waters and the sea floor. Most of the suspended material in seawater is in very small particles^{5,6}. Marine 'snow', consisting of macroscopically visible particles has been seen, however, wherever submersibles have explored. The abundance of deep-sea animals that feed on large food items is much greater than was realised before baited deep-sea cameras⁷ were used, suggesting that some of the sinking particles are large.

The herbivorous zooplankton of upwelling areas and of temperate waters are typically calanoid copepods. Copepods and certain other taxonomic groups of herbivorous zooplankton, by producing faecal pellets make a major contribution to the sinking flux of POC. About 20–30% of the organic carbon of the phytoplankton ingested by such creatures is passed in the faeces of the animals and the faeces are encapsulated in durable, fast-sinking packets which can rapidly transport organic carbon to a great depth. This mechanism was first discovered by radioecologists studying the vertical flux of radionuclides in the ocean⁸. Typical sinking rates of faecal pellets in laboratory experiments are $50\text{--}300 \text{ m d}^{-1}$ (ref. 9).

In coastal temperate waters, traps for intercepting particles have caught largely intact phytoplankton during the spring bloom¹⁰ and largely animal faecal material as the zooplankton assemblage developed to graze down the phytoplankton bloom^{11,12,15}. Trap catches in the Peru upwelling have been dominated either by intact phytoplankton, by zooplankton faecal pellets and moults, or by the faecal material of the anchoveta, *Engraulis ringens*¹³. Catches with faecal pellets are also reported for oligotrophic, deep ocean situations^{5,14,16}. The total flux seems to be approximately proportional to the plankton production in the overlying water. Because of the differential sinking and decomposition rates of large and small particles, the relative importance of faecal pellets as mediators of particulate carbon transport may increase with depth¹⁴.

New production and carbon flux

Primary production in the illuminated surface layer of the ocean—the euphotic zone—can be viewed³ as depending on two different sources of nitrogenous nutrient supply. One is the supply of ammonia, urea and to a lesser extent amino acids and other dissolved organic nitrogen compounds derived from the excretory activities of animals and the metabolism of heterotrophic microorganisms. The food or substrate that fuels these activities, including the source of nitrogen, derives from phytoplankton via the food web. The phytoplankton production resulting from this recycled nitrogen is called regenerated production (Fig. 1). In an ideal closed system the cycling of nutrients through an enclosed food web could continue indefinitely with steady-state standing stocks and fluxes. In the real ocean, however, there are losses such as the flux of sinking faecal material and cast off exoskeletons out of the euphotic

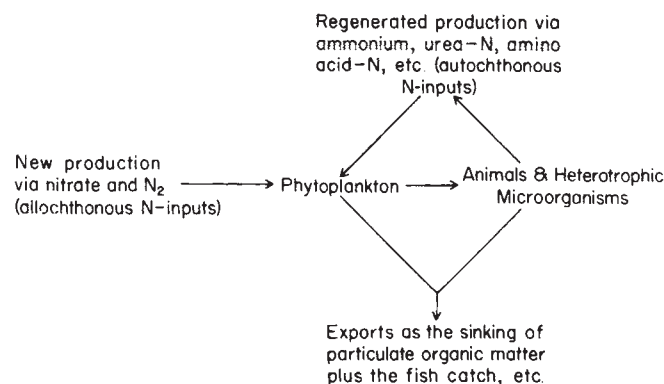


Fig. 1 The production system of the surface ocean, illustrating the concepts of new and regenerated production. Phytoplankton growth is driven by nitrogen inputs of two qualitatively distinct sorts: regenerated and 'new' production. These two pathways leading to phytoplankton production are measured as the phytoplankton assimilation of the various forms of nitrogen using ^{15}N -labelled substrates. This is not possible with other nutrient elements because with carbon or phosphorus, for example, it is not easy to distinguish between autochthonous and allochthonous inputs. To relate new production to export requires that nitrification in the euphotic zone be negligible.

zone to deep water. The fish catch is another example of such a loss⁴, amounting to $<10^7$ tons per year of organic carbon. Seabirds feeding in the ocean but depositing guano on land is another. There is also a concentration gradient of dissolved organic carbon and nitrogen between the surface and deep ocean, the surface values being higher. Rates of physical mixing¹ and concentrations¹⁷ suggest a downward dissolved organic carbon flux of $0.1\text{--}0.3 \times 10^9$ tons C yr^{-1} .

The nutrients lost must be replaced by external inputs to avoid a decline in the productivity of the system. The fossil record shows that production has indeed fallen to low levels in the geological past^{18,19}. Renewal takes place naturally by the injection of nutrients from deep water into the euphotic zone. Although the flux of nitrate from deep water is the predominant method of renewal in the ocean there are other sources: the fixation of molecular nitrogen by free-living and symbiotic blue-green algae (Fig. 1); and the nitrogen from terrestrial and atmospheric sources²⁰. This so called 'new' production, resulting from the assimilation of nitrogen from these sources, can be exported from the productive system without its reducing and, as such, drives both the fish catch and the downward flux of organic matter to bathypelagic and benthic food webs.

To what extent does the sinking flux of particulate organic carbon constitutes a 'sink' for atmospheric CO_2 from fossil fuel burning? Since dissolved inorganic carbon moves upward along with the vertically transported nitrate, in approximately the Redfield ratio²¹ of 106 C atoms: 16 N atoms, only the sinking flux due to new production associated with nitrogen fixation and nutrient inputs from terrestrial and atmospheric sources can be identified as a biologically-mediated transport of atmospheric carbon dioxide to the deep ocean.

Estimation of global new production

The geographical and seasonal data on new production estimates, including measurements of nitrate assimilation and nitrogen fixation, are insufficient to assess global new production directly. Total primary production, however, is quantitatively related to new production (Fig. 2) so that the measurements of total production, based on the photosynthetic assimilation of ^{14}C -labelled CO_2 , can be used to provide preliminary estimates of global new production.

In Fig. 2, the ratio of new production to total production was calculated in terms of nitrogen from the rate of nitrate incorporation (new production) and the sum of nitrate plus ammonium plus an estimate of the incorporation of urea and other organic nitrogen used by phytoplankton (total production). McCarthy²² found urea-N incorporation averaged 28% of the total N incorporation measured as nitrate + ammonium plus urea-N in southern California coastal waters. In the central North Pacific, urea-N incorporation averaged about one-half that of ammonium-N²³. The line of Fig. 2 was not very sensitive to the estimate of organic-N incorporation. Total N incorporation was similar when calculated either as 1.3 (nitrate plus ammonium incorporation, Fig. 2a) or as (nitrate + 1.5 ammonium incorporation, Fig. 2b). Figure 2b shows values for individual stations in the offshore (depth > 300 m) portion of the Southern California Bight, and provides a measure of the station-station variability; it also reveals some interesting exceptions to the expected values.

Data for shallow (< 200 m) inshore waters of the Southern California Bight are not shown in Fig. 2b and they often fall below the line drawn in Fig. 2. This is probably because of the increased supply rate of ammonium from sediments and benthos in the shallow waters. The generality of Fig. 2a is applied here only for deep waters not under the influence of terrestrial runoff or the bottom—for waters where the vertical flux of nitrate is the principal driving force for new production^{24–26}.

Most of the recent estimates of the total ocean primary production are between 20 and 45×10^9 tons C yr^{-1} . To estimate global new production we have used two estimates of total production which distinguish between production over deep water where Fig. 2 might be valid and production in shallow waters where it would not. New production was calculated from these, using Fig. 2a to judge what fraction of total production might be new production. The initial slope of the line of Fig. 2a, for total production $< 200 \text{ g C m}^{-2} \text{ yr}^{-1}$, is described by

$$\text{New/Total} = 0.0025 (\text{Total}) \quad (1)$$

Tables 1 and 2 offer different regional groupings of the production data. New production from the Koblentz-Mishke *et al.* compilation²⁷ and excluding their 'neritic water' category (Table 1) is 18% of total production or 3.4×10^9 tons C yr^{-1} .

Estimates of Table 2 are based on the most recent measurements of total primary production in the oceans²⁸. The estimate of total and new production for waters of depth > 200 m, is the highest of those considered and suggests new production is nearly 20% of total production or about 4.7×10^9 tons C yr^{-1} .

Table 1 Ocean primary production by water type* and estimates of new production based on Fig. 2

	Area ($\times 10^6 \text{ km}^2$)	Primary production ($\text{g C m}^{-2} \text{ yr}^{-1}$) ($\times 10^9 \text{ tons C yr}^{-1}$)		New production (% total) ($\times 10^9 \text{ tons C yr}^{-1}$)	
Oligotrophic waters of the central parts of subtropical halostatic areas	148	25.6	3.79	6	0.24
Transitional waters between subtropical and subpolar zones; extremity of the area of equatorial divergences	83	51	4.22	13	0.54
Waters of equatorial divergence and oceanic regions of subpolar zones	86	73	6.31	18	1.15
Inshore waters	39	124	4.80	30	1.45
Neritic waters	11	365	(3.90)	46	(1.85)
Totals	356		19.1		3.4

* From ref. 27.

Table 2 Ocean primary production in offshore ocean areas* and estimates of new production based on Fig. 2

Ocean	Offshore area ($\times 10^6$ km 2)	Primary production		New production	
		(g C m $^{-2}$ yr $^{-1}$)	($\times 10^9$ tons C yr $^{-1}$)	(% total)	($\times 10^9$ tons C yr $^{-1}$)
Indian	71.0	84	5.96	21	1.25
Atlantic	83.9	102	8.56	26	2.18
Pacific	167	55	9.14	14	1.26
Antarctic	11.8–23.8	325	(3.30)	45	(1.49)
Arctic	13.1	1	(0.13)	0	(0)
Totals	347–359		23.7		4.7

Production over continental shelves and in other areas of water depth <200 m, including coastal upwelling areas, is omitted. Values in parentheses were considered under the <200 m category by Platt and Subba Rao and are omitted from the total estimates.

* From ref. 28.

The polar oceans were not included in the calculations as equation (1) may not apply where growing seasons are short while ambient nutrients are high as in the Antarctic.

The large variation between the estimates of both total and new production for the global ocean leads to the conclusion that new production over the deep ocean may be between 3 and 5×10^9 tons C yr $^{-1}$, excluding the polar oceans. The lower estimate seems more reasonable in view of estimated rates of the decomposition of organic matter and the attendant oxygen consumption and nutrient regeneration rates in the deep water column. For example, if 18% of total production sinks to the deep ocean (Table 1), and 3.0% reaches the sediments³⁰, then about 15% or about 2.8×10^9 tons C yr $^{-1}$ would be decomposed in the water column. This decomposition would, in a homogeneous ocean, require about $10 \mu\text{l O}_2 \text{ l}^{-1} \text{ yr}^{-1}$ on average. Menzel's³⁰ oxygen consumption rates are 9.2–13.4 at 500 m depth and $2.1 \mu\text{l O}_2 \text{ l}^{-1} \text{ yr}^{-1}$ at 4,000 m. The regeneration of

nitrate from organic nitrogen from such a flux would be about $26 \times 10^{-6} \text{ mol m}^{-3} \text{ yr}^{-1}$ based on Redfield's elemental ratios for particulate organic matter²¹ and ignoring losses that could result from the formation of N_2 and N_2O . If the average nitrate concentration of the deep ocean is 30 mmol m^{-3} , its regeneration or formation time would be $\sim 1,200$ yr, a value similar to other estimates of the average residence time of the deep water¹; Table 2 gives 800 yr for this time. If only 1% of the production reached the sediments this time would be decreased to about 1,000 and 720 yr, respectively. Thus the new production estimates of the sinking of particulate organic matter to the deep sea are not inconsistent with other estimates of the rate of decomposition of organic matter. However, this would incorporate^{1,29} a fairly wide range of estimates of such rates and for the flux of particulate organic carbon.

New: total production and nutrient recycling

Figure 2a implies that the extent of nutrient recycling within the euphotic zone varies regionally with the total production rate. If f is the new: total production ratio, then f is the probability that a nitrogen atom is assimilated by phytoplankton via new production and $(1-f)$ is the probability of assimilation via regenerated production. The number of times a nutrient element is recycled in the euphotic zone before sinking out in particulate form (r) is given by $(1-f)/f$. For the central North Pacific (Fig. 2a), $f = 0.05$ and $r = 19$. If $f = 0.5$ (the asymptote of Fig. 2a), $r = 1$. Maximum observed values of f are about 0.8 implying that $r = 0.25$, and only one-quarter of the nutrient is recycled while three-quarters sinks out after being assimilated by phytoplankton. This could occur only if intact phytoplankton accounted for a major fraction of the sinking POC, as observed at the end of a spring bloom^{10,11}, and transiently in the Peru upwelling¹³. The ratio f thus provides qualitative information on the nature of the sinking POC as well as quantitative information on the extent of nutrient recycling within the euphotic zone.

Broecker's geochemical model for the global ocean¹ suggests that an element such as nitrogen is recycled about 10 times in the surface layer before sinking out. Thus $r = 10$, $f \approx 0.1$ and new production is $\sim 10\%$ of the total primary production. Slightly higher values are suggested in Tables 1 and 2 where new production may be about 18 or 20% of total production, respectively.

Checking the validity of the approach

Broecker *et al.*³¹ estimated a mean upwelling rate of 27 m yr^{-1} for the equatorial Atlantic between 15° S and 15° N latitude. No measurements of the new: total production ratio have been made there to our knowledge. If the deep-water nitrate concentration is $\sim 15 \text{ mmol m}^{-3}$ the nitrate flux to surface waters would be $405 \text{ mmol m}^{-2} \text{ yr}^{-1}$. From the Redfield ratio, this would support a photosynthetic new production rate of $32.2 \text{ g C m}^{-2} \text{ yr}^{-1}$; from equation (1) the total primary production would then be $(32/0.0025)^{1/2} = 113 \text{ g C m}^{-2} \text{ yr}^{-1}$. Suschenya and Finenko's³² earlier measurements with the ^{14}C method of primary production in this area gave for the (Northern Hemisphere) fall season an average of $201 \text{ g C m}^{-2} \text{ yr}^{-1}$. Earlier data for the spring season averaged 88 g C m^{-2} . If annual production were the mean of these values, $145 \text{ g C m}^{-2} \text{ yr}^{-1}$, this would give fair agreement between the calculated and measured values. Although the result is encouraging, the spatial and temporal variation in the total production measurements suggests that care should be taken in estimating regional averages from a few scattered stations.

Conclusions

New production depends on mixing and vertical advective processes associated with the circulation. Total production also depends on new production and is roughly proportional to it at low to moderate production rates²⁶. Thus over geological time climatic changes that reduced vertical mixing and advection must have greatly affected the planktonic production process.

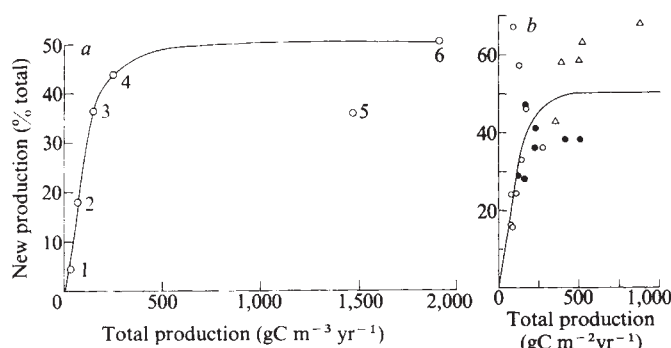


Fig. 2 a, New production as % of the total primary production versus total production for various ocean areas: (1) Central North Pacific, (2) eastern Mediterranean Sea, (3) Southern California Bight, (4) eastern Tropical Pacific, (5) Costa Rica Dome, and (6) Peru upwelling. Total production was measured by the ^{14}C method. New: total production ratio measurements are based on the assimilation of ^{15}N -labelled nitrate and ammonium. Assimilation of urea and other organic-N was assumed to be either 30% of the total N assimilation²² or one-half of the ammonium assimilation rate²³. Results were similar with either correction. No correction was applied for new production as molecular nitrogen fixation as this is assumed to be small^{33,34}. Values are regional averages from Dugdale³⁵ (points 2, 4, 5, 6) and from this laboratory²⁶ (points 1, 3). The total production rates, from ^{14}C measurements of photosynthesis, are not annual averages but are daily rates $\times 365$ for particular sets of measurements. For example, the annual production of the Peru upwelling area is probably less than the $1,900 \text{ g C m}^{-2} \text{ yr}^{-1}$ shown here. b, New: total production ratio versus total primary production at individual stations in the Southern California Bight. Nearshore stations in water depth <300 m are omitted. New: total production ratio was calculated as nitrate incorporation rate: (nitrate + 1.5 ammonium incorporation rates). For details see ref. 26. Symbols represent different cruises.

Note that both too little and too much mixing will reduce production, the latter by increasing the critical depth below that which will support net growth of phytoplankton. How increasing levels of atmospheric CO₂ from fossil fuel burning and forest destruction will affect climate-related ocean mixing and hence

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future plankton production and fisheries harvests is not clear.

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Molecular structure of a left-handed double helical DNA fragment at atomic resolution

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The DNA fragment d(CpGpCpGpCpG) crystallises as a left-handed double helical molecule with Watson-Crick base pairs and an antiparallel organisation of the sugar phosphate chains. The helix has two nucleotides in the asymmetric unit and contains twelve base pairs per turn. It differs significantly from right-handed B-DNA.

MANY biological and chemical phenomena are analysed in the context of the molecular structure of the Watson and Crick¹ double helix of DNA; the B form of DNA is believed to be the predominant form in biological systems. Alternative conformations of the double helix have also been discussed, including modifications of the helical parameters with altered tilting of the bases. Most of our knowledge about the molecular structure of DNA arises from X-ray diffraction studies of DNA fibres². This technique has considerable constraints—the amount of experimental data is small, so the interpretations have to be limited, and atoms are not resolved. To understand the structure of double helical DNA in more detail, it is desirable to crystallise DNA fragments of fixed sequence and solve their three-dimensional structure at atomic resolution. Here we report the crystallisation and structural analysis of a double helical fragment of DNA containing six base pairs with the sequence d(CpGpCpGpCpG). DNA with an alternating dG–dC sequence is of considerable interest as it is known to exist in two distinctly different conformations which are stable in both low or high salt solutions³. The crystal structure of this self-complementary hexamer has been solved to atomic resolution at 0.9 Å. The structure is found to be a left-handed double helix with a novel three-dimensional structure which is quite distinct from the familiar right-handed helical B form of DNA.

Crystallisation and structure solution

The ammonium salt of the deoxy hexamer d(CG)₃ was prepared by a slight modification of the recently developed^{4,5} phosphotriester approach. The crystals were grown from a solution containing 30 mM sodium cacodylate buffer (pH 7.0), 10 mM spermine tetrachloride, 15 mM MgCl₂ and 2 mM d(CG)₃ using the vapour diffusion method as developed for tRNA crystallisation⁶. The precipitating agent was 5% isopropanol. Crystals grew in the form of truncated rectangular plates over a period of 3 weeks and crystals measuring up to 0.7 × 0.7 × 0.5 mm were obtained for X-ray diffraction analysis. Crystals were mounted in a sealed glass capillary together with a droplet of mother liquor. The crystal was orthorhombic with space group P2₁2₁2₁ and cell dimensions *a* = 17.88, *b* = 31.55 and *c* = 44.58 Å. The *h*01 precession photograph revealed strong reflections in the region 3.4–3.7 Å along the *c* axis which suggested that the molecules were aligned with their bases perpendicular to the *c* axis. From the unit cell dimensions, we estimated that there would be one duplex containing 12 nucleotides in the asymmetric unit. The crystals were highly ordered and complete three-dimensional data were collected to a resolution of 0.9 Å using a Picker diffractometer.

The structure was solved by the method of multiple isomorphous replacement using three different heavy atom derivatives, Ba²⁺, Co²⁺ and Cu²⁺. Phase determination was carried out using the method of Blow and Crick⁷, yielding a mean figure of merit $\bar{m} = 0.76$ to 2 Å resolution and the 'best' Fourier map was calculated⁸. The structure was traced from this electron density map and rough atomic coordinates were measured directly from the map. The structure was refined using the Konnerth-Hendrickson refinement procedures⁹. Several cycles of difference Fourier and sum function Fourier ($2F_0 - F_c$) maps were calculated in order to locate all the atoms of the molecule as well as 62 water molecules, 1 hydrated Mg²⁺ ion and 2 spermine molecules. The mass of the asymmetric unit is

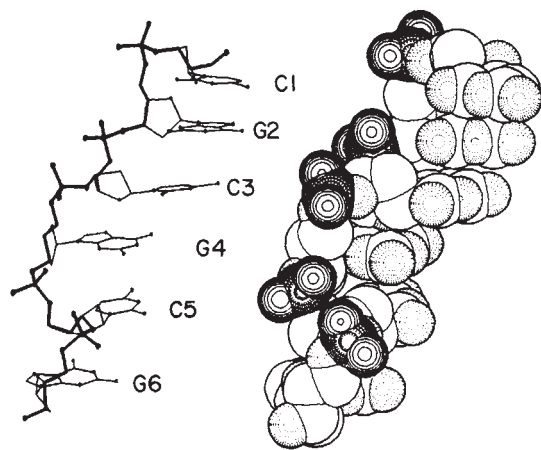


Fig. 1 Diagram showing the molecular structure of the deoxyhexanucleoside pentaphosphate which makes up one half of the duplex in the asymmetric unit. Skeletal and van der Waals views are shown together with the numbering system. The stacking of the bases as well as the stacking of G4 guanine on the deoxyribose of C3 can be seen clearly in both diagrams. The phosphate groups pursue a zig-zag course along the polynucleotide chain. Hydrogen atoms are not included in the diagrams.

approximately 5,000 daltons. The current residual index (R factor) is 14% at 0.9 Å resolution using 15,000 reflections ($>2\sigma$). All atoms were refined isotropically and hydrogen atoms were not included in the refinement. A full description of the crystallographic methods will be published elsewhere, and further refinement is in progress.

A surprising structure

The structure of $d(CG)_3$ has some of the familiar features of B-DNA. It is an antiparallel double helical fragment with Watson-Crick base pairing between the guanine and cytosine bases. However, unlike B-DNA, the structure has a left-handed helical sense. Even though the oligomer has only six base pairs, the structure has considerable internal regularity. Furthermore, the disposition of the molecules within the crystal lattice is such that symmetry related hexamers stack one on another so as to closely approximate an infinite polymer of alternating cytosine-guanine sequence. The details of the structure presented here refer to the hexamer itself, while comparison with B-DNA refers to the left-handed polymeric DNA seen in the crystal packing.

Figure 1 shows a view of one half of the duplex in the asymmetric unit with the six residues C1 to G6. The other half contains residues C7 to G12, similarly organised in a helical form.

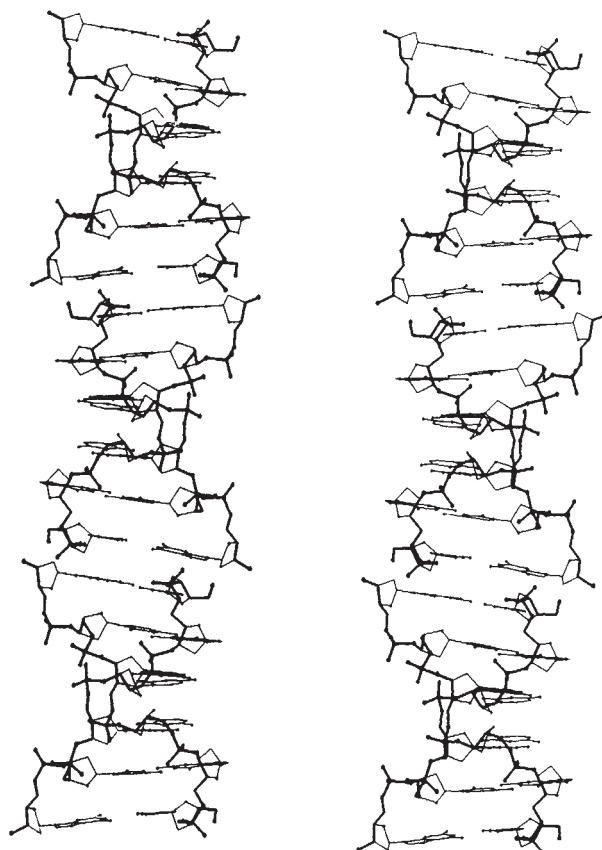
As seen in Fig. 1, the six bases in the half molecule are aligned parallel to each other. However, the backbone is rather unusual. This can be seen clearly in the van der Waals diagram which shows that the phosphate groups pursue a zig-zag course along the backbone. Furthermore, the sugar residues have an alternating orientation in that the dG residues have the deoxyribose O1' pointing up while the dC residues have the deoxyribose O1' pointing down. Because of this alternation, the repeating unit in the helix is not a mononucleotide as in B-DNA, but a dinucleotide. Figure 2 is a stereo view of three molecules stacked along the c axis. These appear to form a continuous double helix, even though the helix is interrupted by the lack of a phosphate group on the terminal residues. The helix contains 12 base pairs per turn or, more specifically, six dimer residues. Each dimer of dCpGp (or dGpCp) is related to the next by a rotation of -60° . The double helix has base pairs which are tilted 7° from the helix axis. The stereo drawing illustrates the two different types of phosphate positions along the backbone. These differences are associated with differences in the glycosidic conformation between dG and dC residues. There are two general positions which a base can occupy relative to the sugar residue, *anti* and

syn. In this structure, bases of the dC residues are in the *anti* conformation as in B-DNA while the dG residues are in the *syn* conformation. The guanine *syn* conformation is associated with a pucker for the sugar residues which is $C3'$ *endo* for the two dG residues in the middle of the strand while the terminal dG has a $C2'$ *endo* conformation. The torsion angle around the $C4'-C5'$ bond is *gauche-trans* for the dG residues. The dC residues are in the *anti* conformation, the sugar has the $C2'$ *endo* pucker and the $C4'-C5'$ bond is *gauche-gauche*. Thus, the dC residues have the conformation found in B-DNA, whereas the internal dG residues have an unusual rotation of the base around the glycosidic bond, an unusual pucker of the deoxyribose ring, and an unusual conformation about the phosphate residue. In looking at this left-handed helix (Fig. 2), it is apparent that the ribose-phosphate backbone follows a zig-zag course resulting from the alternating residue conformations. Accordingly, we propose to call this Z-DNA.

The double helix is internally regular

Although the bases are stacked in a manner that seems similar to that found in B-DNA, there are considerable differences, as illustrated in Fig. 3. The $d(CG)_3$ double helix has six levels of base pairs, base C1 is paired to G12, G2 to C11, and so on. These are viewed pairwise, down the helix axis, in Fig. 3. Thus, 1-12/2-11 shows the upper base pair drawn in heavy lines superimposed on the next lower base pair drawn in lighter lines. The column on the left shows the base stacking of the first pair over the second, the third over the fourth and the fifth over the sixth; all of which contain dCpG sequences. The dCpG sequence of B-DNA is shown at the bottom¹⁰. The dCpG base pairs in

Fig. 2 Stereodrawing of three molecules in the crystal lattice as they are stacked along the c axis in what looks like a continuous double helix. However, a phosphate group is absent every sixth residue along the chain. Successive base pairs along the helix have the deoxy ribose rings orientated so that O1' is alternately pointing down or up, and the same orientation is found in both sugar residues across the base pair. This indicates that the helix asymmetric unit is two nucleotides rather than one as in B-DNA.



Z-DNA are not stacked on each other directly but rather are associated with a lateral translation of almost 7 Å relative to each other so that they present a sheared appearance with very little rotation from one base pair to the next along the chain. The cytosine of the upper base pair is partially stacked on the cytosine of the lower base pair. Instead of being stacked on the bases, the guanines are stacked upon the O1' oxygen atoms of the preceding deoxyribose residues. This figure also shows the *syn* conformation of the dG residue. What is striking in Fig. 3 is the fact that all three of the stacking diagrams for the dCpG sequences are similar to each other, as the double helical fragment is internally regular. However, this regularity is not imposed by crystallographic constraints of the lattice but appears to be a direct consequence of the double helical structure of the molecule. Note, however, that the regularity is not exact. There are minor perturbations associated with the distribution of various ions in the lattice.

The solid circle in Fig. 3 shows the position of the helix axis which is located outside of the base pair close to oxygen O2 of cytosine in what would correspond to the minor groove of the Z-DNA helix. This is in contrast with that of B-DNA (shown at bottom) in which the helix axis passes through the base pair.

In Fig. 3, the sense of the sugar phosphate chains is constant for both Z-DNA and B-DNA. Thus, the sugar phosphate backbone is going 5' to 3' away from the reader on the left-hand side of all diagrams. Note that the minor groove of Z-DNA is below the base pairs, whereas in B-DNA, the minor groove is above the base pairs. There has thus been a reversal of the position of the minor groove of Z-DNA relative to B-DNA. Furthermore, the major groove does not exist as a groove in Z-DNA but has been transformed into a convex surface on the upper part of the molecule as drawn in Fig. 3.

The column on the right of Fig. 3 shows the stacking of dGpC residues. There is considerable stacking overlap of the bases on each other in a manner which is somewhat similar to that of B-DNA. Furthermore, the distance between the phosphate groups across the helix (15 Å) is close to that of B-DNA (17.5 Å), while the corresponding distance in dCpG residues (12.5 Å) is somewhat shorter. In contrast to the dCpG sequence, there is a considerable rotation associated with the adjacent base pairs of dGpC along the helix (see Table 1). At the lower right, the position of base pair G6-C7, at the bottom of one helix, is shown relative to the position of base pair C1'-G12' of the helix immediately below it in the crystal lattice. This packing is virtually identical to the internal stacking of the dGpC residues shown above it. Thus, the helix maintains continuity across the two molecules even though it is missing two phosphate residues at the end. There is a small perturbation at the end of the molecule, as mentioned above. Guanosine residues 6 and 12 have the *syn* conformation of the sugar residues but have the C2' *endo* sugar pucker instead of C3' *endo* as the internal guanosines. This is probably due to the absence of the phosphate linkage.

Figure 3 shows that the imidazole rings of guanine residues are projected well away from the centre of the Z-DNA molecule and provide part of the convex surface of the molecule. Atoms N7 and C8 are exposed to the external solvent as a direct consequence of the *syn* conformation of the guanosine residues.

A slimmer DNA helix

An end view of Z-DNA compared to B-DNA is shown in Fig. 4. In both of these figures, one complete turn of the double helix is shown. This consists of 10 base pairs of B-DNA with an alternating G-C sequence and 12 base pairs of Z-DNA. To illustrate the difference in position of the bases, all of the guanine residues in one chain are shaded. It can be seen that there is an approximate 6-fold symmetry of Z-DNA with the guanine bases largely found in the outer part of the molecule. In contrast, there is a 5-fold symmetry of the B-DNA with the bases clustered near the centre of the molecule. (The symmetry is 5-fold for G alone, but 10-fold for the regular backbone.) Furthermore, Z-DNA is a slightly smaller helix with a diameter of 18 Å compared to the

20 Å diameter of B-DNA. The projection of B-DNA has exact symmetry as it is derived from a model which assumes that each base pair is found in a symmetrically identical position. The Z-DNA diagram is a projection down the complete 44.6 Å *c* axis of the crystal. It does not have the regularity of the B-DNA model, partly due to the fact that two phosphate groups are missing from the helix.

Z-DNA has novel symmetry

Figure 5 shows van der Waals side views of Z-DNA and B-DNA. The Z-DNA molecule looks like a cylinder with the bulk of its mass making up the walls. Z-DNA is a helix with six units per turn, where each unit has two base pairs. Accordingly, the view of the helix repeats every 60° about the axis. Figure 5 shows two views of Z-DNA 90° apart, but because of the 6-fold

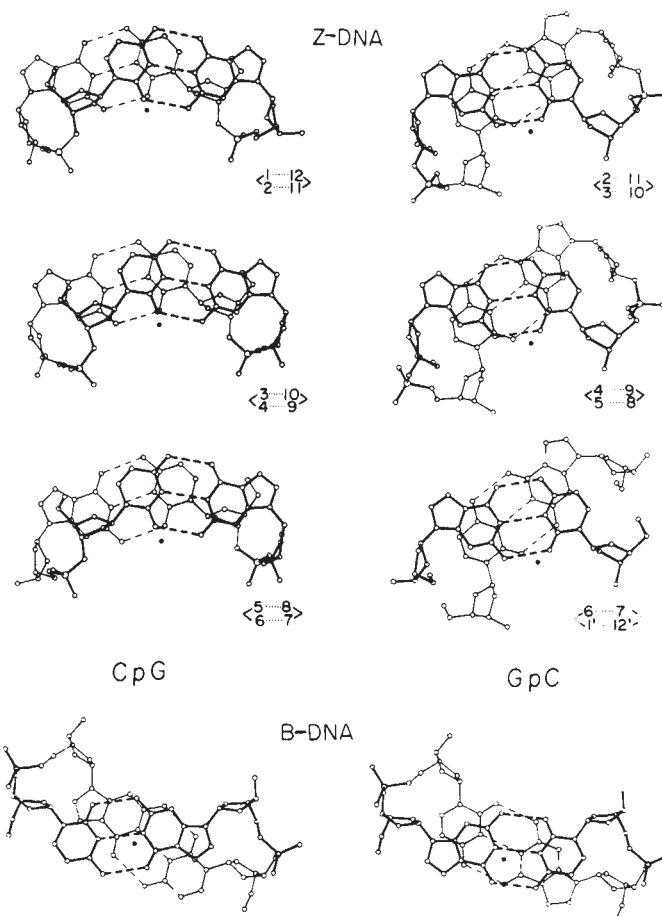


Fig. 3 A stacking diagram illustrating the overlap of successive bases in the double helical fragment. The molecule contains nucleotides 1 to 6 in one chain and 7 to 12 in the other chain such that C1 is hydrogen bonded to G12, G2 to C11, and so on. The base pair drawn with a heavier line stacks above the base pair drawn with a lighter line. Three different overlaps are shown in the left-hand column which represents the stacking of dCpG sequences between levels 1 over 2, level 3 over 4, and level 5 over 6. These demonstrate a remarkable regularity in the internal portions of the Z-DNA double helix. Nonetheless, close inspection reveals that this regularity is not exact. The three columns on the right show the overlaps of level 2 over 3, 4 over 5 and the bottom base pair overlapping on the top base pair of the molecule immediately below. These dGpC overlaps are also very similar to each other including the stacking between molecules. For comparison the overlaps of the stacking interaction of dCpG and dGpC are shown for B-DNA at the bottom. The orientation of the ribose phosphate chains is the same in all of these diagrams. Major and minor grooves are defined in B-DNA in terms of their relationship to the base pairs. Note that the minor groove in B-DNA is found at the top of the B-DNA diagrams but at the bottom of the Z-DNA diagrams. The helix axis is shown by a solid circle.

symmetry, these are equivalent to two views 30° apart. B-DNA at the right has 10 base pairs per turn, accordingly, it looks the same every 36° about the axis. Only one view is shown, as the two views 18° apart are not sufficiently different from each other.

The double helix of B-DNA in Fig. 5 is that of a continuous polymer whereas the double helix of Z-DNA is simply a graphic representation of the contents of the unit cell as viewed from two different angles. However, the contents of the unit cell are very close to those of a continuous double helix. The continuous double helix of Z-DNA has symmetry properties which are similar but not identical to those of B-DNA. B-DNA has two kinds of 2-fold or dyad axes perpendicular to the helix axis which arise because the two backbone chains are antiparallel. These dyad axes are found both in the plane of the base pairs and between each of the base pairs. The Z-DNA backbone is composed of alternating conformations. Accordingly, the dyad axis in the plane of the base pair is lost but the dyad axis between the base pairs is retained. These reflect the fact that the two backbone chains are still antiparallel even though the conformation is different in successive residues along the chain.

One of the striking features associated with Z-DNA is the alternating position of the phosphate groups. This is emphasised in Fig. 5 by drawing a heavy black line from phosphate to phosphate residue along the chain. The heavy line is drawn through all phosphates, including the missing one in the Z-DNA structure. These lines are smooth, continuous and right-handed in B-DNA but follow a zig-zag, discontinuous left-handed course in Z-DNA. Z-DNA has a minor groove which is considerably deeper than the minor groove of B-DNA. The major groove is absent and is more or less everted relative to B-DNA. In B-DNA, the major groove is a concave surface with the base pairs at the bottom, while the analogous part of Z-DNA is a rounded convex surface made up of the base pairs with the imidazole residues of guanine prominently exposed on the surface.

Z-DNA is thinner and more extended than B-DNA. Twelve base pairs are found in $44.6 \text{ \AA}$, whereas B-DNA will accommodate thirteen base pairs in that same distance, because the average distance along the axis is $3.7 \text{ \AA}$ for the base pairs. Although the bases are still stacked $3.4 \text{ \AA}$ apart, the $3.7 \text{ \AA}$ figure is associated with the fact that the base pairs are tilted 7° to the axis and sheared in such a way that the actual distance along the helix axis is $3.7 \text{ \AA}$. Z-DNA looks like a cylinder and has less of a grooved appearance than B-DNA because it has only one narrow groove.

The Z-DNA molecule is constructed largely of a band of material wrapped around an axis which is not occupied by atoms. This left-handed wrapping creates a deep groove extending all the way down to the axis of the molecule. The groove has a serrated edge of phosphate groups which are produced by the zig-zag character of the deoxyribose phosphate chain. The phosphate groups are $8.5 \text{ \AA}$ apart between the two edges and the groove itself is approximately $9 \text{ \AA}$ deep as it extends to the axis of the $18 \text{ \AA}$ diameter molecule. The phosphate groups are neutralised by cations and their organisation will be described elsewhere. A significant amount of space is

occupied by the helical groove which wraps around the molecule. The groove can be seen in the stereodigram in Fig. 2, but the depth of the groove is less apparent in the van der Waals diagram of Fig. 5. An idealised model of polymeric Z-DNA has been constructed and will be described elsewhere.

Detailed knowledge of DNA structure

Most of our knowledge on the molecular structure of DNA derives from fibre diffraction studies, which provide information on the general organisation of the sugar phosphate chains and the bases but are limited in resolution, and from X-ray diffraction studies of crystalline nucleotides and nucleosides, which provide information about the geometry of the individual components. Until now there has been no detailed information concerning the macromolecular fine structure of DNA molecules. We have some knowledge about the organisation of helices. The first studies of this type came from fragments of RNA double helices. The dinucleoside phosphates of rApU (ref. 11) and rGpC (ref. 12) formed double helical fragments in a crystal lattice and provided detailed information about helical RNA fragments. Similar information for DNA was obtained in the structure of the tetramer dpApTpApT (ref. 13) which crystallised in a form with right-handed helical segments containing two base pairs each. Information about extended macromolecular helical structure comes from the structure of yeast phenylalanine transfer RNA which has been refined at $2.5 \text{ \AA}$ resolution¹⁴⁻¹⁷. An atomic resolution study has been reported for a helical fragment of DNA containing two base pairs with an intercalator inserted between them¹⁸. However, segments of a helix with only two base pairs raise concerns about end effects in the molecule. The present study is the first to provide information about a macromolecular segment of DNA.

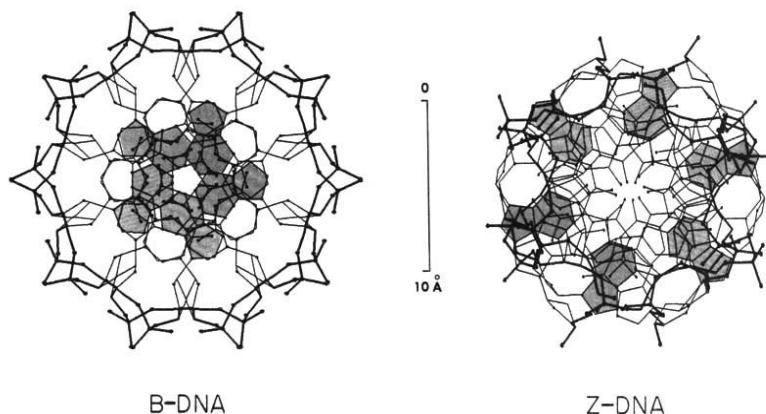
Several alternative models have been proposed for the structure of DNA. Some of these involve 'side-by-side' structures in which the DNA alternatively changes from right-handed to a left-handed form^{19,20}. The structural principles found in Z-DNA differ considerably from those proposed for various left-handed forms of DNA. The essence of the Z-DNA structure is related to conformation differences in the alternating guanine and cytosine residues.

The present structure provides much detail on the organisation of this left-handed form of DNA. This information is a consequence of the atomic resolution data about the molecule itself, but also concerns the organisation of the many water molecules and ions found in the crystal structure. We are thus faced with the interesting paradox that we now have more detailed information on the structure of Z-DNA than we do of the familiar B-DNA which is believed to be the predominant form in biological systems!

Two forms of DNA

Pohl and Jovin³ were the first to describe two conformations of DNA which were found in a sequence containing alternating guanine and cytosine residues. They discovered that raising the concentration of either NaCl or MgCl_2 produced a dramatic,

Fig. 4 End views of B-DNA and Z-DNA are illustrated in which the guanine residues of one strand have been shaded. The Z-DNA figure represents a view down the complete *c* axis of the crystal structure encompassing two molecules. The shaded guanine residues illustrate the approximate 6-fold symmetry. The imidazole part of the guanine residue forms a segment of the outer cylindrical wall of the molecule together with the phosphate residues. The B-DNA figure represents one full helix turn. In contrast to Z-DNA, the guanine residues in B-DNA are located closer to the center of the molecule and the phosphates are on the outside.



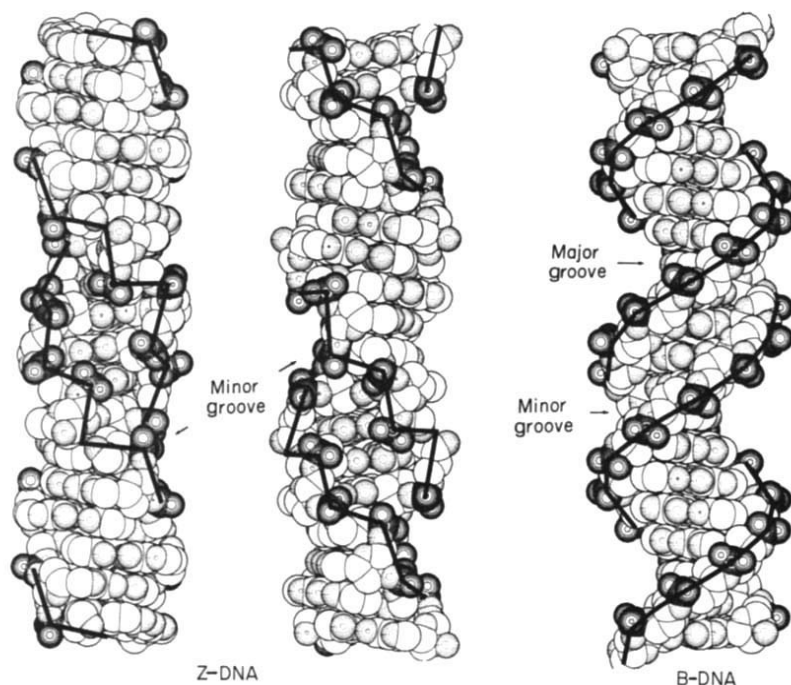


Fig. 5 Van der Waals sideviews of Z-DNA and B-DNA. Two views of Z-DNA are shown which are 30° apart in orientation about the helix axis. The irregularity of the Z-DNA backbone is illustrated by the heavy lines which go from phosphate to phosphate residues along the chain. This includes positions where the phosphate residues are missing in the crystal structure but would be occupied in a continuous double helix. The minor groove in Z-DNA is quite deep extending to the axis of the double helix. In contrast, B-DNA has a smooth line connecting the phosphate groups and two grooves, neither one of which extends into the helix axis of the molecule.

cooperative change from one form to another which is monitored most readily by changes in the circular dichroism (CD). The CD pattern inverted almost completely. This provided evidence for a high salt and a low salt conformation. The conformational change can also be induced by ethanol^{21,22}. Furthermore, adding an intercalator changes the high salt conformation into the low salt conformation²³. Pohl and Jovin also provided evidence that the low salt form was the familiar B-DNA³. More recent magnetic resonance studies of high and low salt forms of this polymer reveal that the high salt form has two ³¹P resonances while the low salt has only one resonance which is also found in B-DNA²⁴.

The question we naturally must ask is what form of the molecule do we see in Z-DNA? The crystal was set up in a salt environment which was approximately physiological with very little salt compared to the high concentration required to convert the alternating polymer to the high salt form. Nonetheless, the Z-DNA structure shows two distinct kinds of phosphate environment. The marked differences between the two phosphodiester linkages in dCpG and dGpC suggest that this is the origin of the two peaks seen in the high salt ³¹P resonance spectrum. The high salt assignment is further strengthened by analysis of the PMR spectrum of the high salt form²⁴. It is possible that a small amount of Z-DNA may exist in a solution which is predominantly B-DNA at low ionic strengths. However, crystallisation of the molecule in the Z form produces a gradual conversion of the solution during the crystallisation process. It is also possible that the presence of the spermine molecule may have facilitated this conversion. The fact that the phosphate groups are closer to each other on two sides of the minor groove in Z-DNA than in B-DNA suggests that the structure would be stabilised in solution by the presence of high concentrations of salt to mask phosphate repulsions.

X-ray diffraction patterns have been taken of fibres of poly(dA-dT) · poly(dA-dT) and poly(dG-dC) · poly(dG-dC) as well as poly(dI-dC) · poly(dI-dC) (refs 25, 26). These fibres produce a diffraction pattern, called D-DNA, with eight residues per turn. In the initial analysis of the alternating poly(dI-dC) polymer one interpretation suggested that it might be a left-handed helix²⁶. However, it has dimensions which differ significantly from those which we observe for Z-DNA. Thus the relationship between the fibre diffraction patterns and Z-DNA is not clear at present. The form of Z-DNA which we see in the crystal has 12 residues (six dimer pairs) per turn of the helix. The symmetry of the lattice does not put any constraints on the

molecule, thus there is reason to believe that the present form of the molecule may represent the stable form. It is unclear whether this helical packing can be modified in a polymer. We cannot rule out the possibility that the number of base pairs per turn could change somewhat although it is hard to believe that the structure could be tightened to the point where there would be eight residues per turn in the helix as is proposed for D-DNA.

Topological relationship between Z-DNA and B-DNA

Both B-DNA and Z-DNA have the form of a double helix in which the two deoxyribose phosphate chains have opposite polarity; the chains are related by dyad axes as discussed above. However, there are some significant differences between the two molecules, as noted in Table 1. If one faces the minor groove of a B-DNA model, the deoxyribose phosphate chain on the right has an orientation such that 5' to 3' is going down. If one faces the minor groove of Z-DNA, the deoxyribose-phosphate chain on the right has the opposite polarity. How then does one convert from B-DNA to Z-DNA? Figure 6 is drawn to illustrate the manner in which this conversion can be understood. The major difference between the two molecules is related to the orientation of the base pair relative to the sugar phosphate chain. In effect, the base pairs in Z-DNA are flipped over so that they are 180° away from the position they have in B-DNA. This is illustrated in Fig. 6 by shading one surface of the base pair and leaving the other surface blank. In B-DNA, all the upper surfaces are blank. Conversion to Z-DNA may be associated with an initial separation of the base pairs, a rotation followed by a rejoining of the base pairs. Rotation about the glycosidic bond occurs only for the guanine residue resulting in the *syn* conformation. The entire cytidine residues rotates, base plus sugar, so that it remains in the *anti* conformation. Rotation of the sugar residues of cytidine is one of the elements producing the characteristic zig-zag of the sugar phosphate backbone. The difference between dG and dC residues is not illustrated in Fig. 6 for the sake of simplicity.

We have carried out model building studies of Z-DNA and B-DNA and have asked whether it is possible for these to join. We believe that it is possible to join, but only in a specified way. As shown in Fig. 3, the phosphate-phosphate distance across the helix is larger for the dGpC segment than for dCpG. The phosphate distance across the dGpC segment (15 Å) is close to

that found for B-DNA (17.5 Å). One can thus make a joining to B-DNA at that point but not at the middle of a dCpG segment. This suggests that a segment of Z-DNA in the middle of B-DNA is likely to involve an even number of nucleotides. However, it is important to note that in Z-DNA relative to B-DNA, the base pairs are stacked on opposite sides of a line joining the two phosphate groups across the helix. This means that the juncture between B-DNA and Z-DNA will have a stacking discontinuity which might result in a kinking of the molecule. Figure 6 is drawn in such a way to suggest a discontinuity of stacking.

There is some evidence which suggests that Z-DNA segments can exist in naturally occurring DNA. Alkylation of guanine residues in DNA by mitomycin C results in changes in the CD which are similar to the changes found in the high salt form of the alternating dC-dG polymer^{27,28}. This suggests that segments of Z-DNA can be found in the middle of B-DNA when driven by the impetus of guanine alkylation. CD studies of DNA in high salt solutions also suggest that segments of the molecule may convert to Z-DNA²⁹. Interestingly, self-annealing of single-stranded circular plasmid DNA results in the formation of a double helical structure which appears to be intact over 70% of its length³⁰. This is also associated with changes in the CD which are consistent with the formation of left-handed segments of Z-DNA in the annealed circular DNA. In this case, the topological constraints of annealing single-stranded circles with each other has forced the establishment of left-handed segments which may adopt the Z-DNA conformation as judged by the changes in the CD. The relationship between the observed inversion of the CD and the formation of Z-DNA structures, as opposed to any other left-handed helical structure, has to be established more securely in order to draw firm conclusions.

Is the Z-DNA structure strictly limited to alternating guanine and cytosine sequences? At present, we cannot answer with certainty. From looking at the structure, it appears that it could also accommodate any alternating purine and pyrimidine sequences, as the substitution of an AT base pair for a GC base pair would not perturb the structure greatly. There may be a slight bias, however, against the introduction of AT for GC pairs in Z-DNA. A hydrogen bond is found between the guanine amino group N2 and a water molecule which in turn is hydrogen bonded to one of the phosphate oxygens at the edge of the

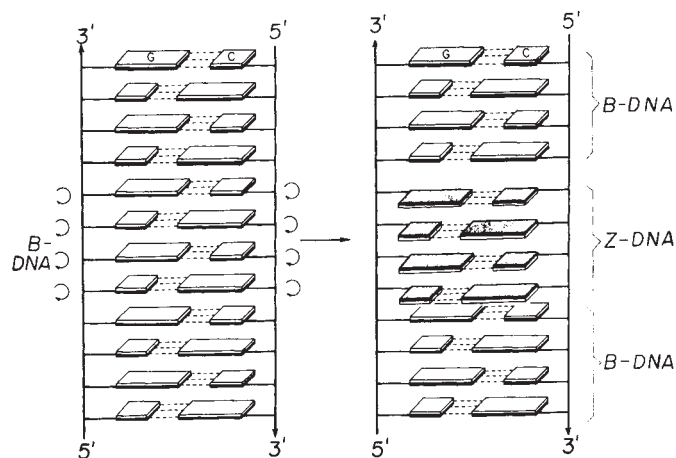


Fig. 6 A diagram illustrating the change in topological relationship if a four base pair segment of B-DNA were converted into Z-DNA. This conversion could be accomplished by rotation of the bases relative to those in B-DNA. This rotation is shown diagrammatically by shading one surface of the bases. All of the dark shaded areas are at the bottom in B-DNA. In the segment of Z-DNA, however, four of them are turned upwards. The turning is indicated by the curved arrows. Rotation of the guanine residues about the glycosidic bond produces deoxyguanosine in the *syn* conformation while for dC residues, both cytosine and deoxyribose are rotated. The altered position of the Z-DNA segment is drawn to indicate that these bases will not be stacking directly on the base pairs in the B-DNA segment.

Table 1 Comparison of helices of B- and Z-DNA

	B-DNA*	Z-DNA†
Helix sense	Right-handed	Left-handed
Residues per turn	10	12 (6 dimers)
Diameter	~20 Å	~18 Å
Rise per residue	3.4 Å	3.7 Å
Helix pitch	34 Å	45 Å
Base pair tilt	6°	7°
Rotation per residue	36°	-60° (per dimer)
Glycosidic torsion angle		
Deoxyguanosine	<i>Anti</i>	<i>Syn</i>
Deoxycytidine	<i>Anti</i>	<i>Anti</i>
Sugar pucker		
Deoxyguanosine	C2' <i>endo</i>	C3' <i>endo</i>
Deoxycytidine	C2' <i>endo</i>	C2' <i>endo</i>
Distance of P from axis		
dGpC	9.0 Å	8.0 Å
dCpG	9.0 Å	6.9 Å

* Data from ref. 10.

† Average values are given and end effects are excluded.

groove. This water molecule is likely to be tightly bonded and may serve to stabilise the structure. However, its significance is unclear and further experiments are needed to determine whether it is possible to form Z-DNA with any alternating purine-pyrimidine sequence. In this regard, it should be noted that *syn* conformations for pyrimidine residues are somewhat less favourable than they are for purine residues, but still a number of molecules have been crystallised with pyrimidine residues in the *syn* conformation. Thus the question of the relationship of the Z structure to alterations in sequence will have to be examined further.

We are equally uncertain about the relevance of the Z-DNA structure to RNA with an alternating guanine and cytosine sequence. The orientation of the sugar is such that the C-H on carbon 2' is projecting out away from the centre of the molecule. It could thus accommodate a ribose residue as well as a deoxy residue. The energy difference between the two forms of sugar pucker for the deoxyribose furanose ring is rather small, but the energy difference may be somewhat greater for ribose residues^{31,32}. Accordingly, this may make the structure less stable for ribonucleotides than for deoxyribonucleotides. However, the relationship between the Z structure and ribonucleotides has to be investigated further in view of existing experimental data^{33,34}.

Biological implications of Z-DNA

The Z-DNA structure represents a stable alternative conformation of DNA with a unique sequence. We believe that it is likely to be used in biological systems at one point or another. Z-DNA requires a higher concentration of cations than B-DNA. However, proteins which bind to nucleic acids are known to contain large numbers of basic residues so that some of these proteins may convert segments of B-DNA into Z-DNA *in vivo*. This question can now be attacked experimentally especially in view of the increased accessibility of the guanine N7 and C8 positions in Z-DNA segments. Z-DNA is an outstanding example in which a substantial conformational change is induced in the DNA backbone as a consequence of a specialised sequence of nucleotides. The specificity of a protein binding to a DNA segment could be very great where this structural conversion is stabilised by the presence of the protein. The ready availability of cloned segments of DNA of defined sequence and proteins which bind to DNA will certainly facilitate the investigation of conformational changes of this type.

If one looks through biologically active sequences, one is struck by the fact that occasionally nature has preserved alternating sequences of guanine and cytosine residues. For example, there is a highly conserved segment in rodent parvoviruses near the origin of replication which contains a total of 20 alternating G and C residues in two segments³⁵. Furthermore, a segment of

the histidine D gene of *Salmonella* has a mutational 'hotspot' which occurs at a segment of eight alternating GC residues³⁶. We can now ask whether these sequences are associated with Z-DNA structures.

There are many agents which are specific for guanine alkylation in the O6, N7 or C8 position. Several of these are highly active carcinogens, such as *N*-acetoxy-*N*-2-acetylaminofluorene, which alkylates in the C8 position, and nitrosamines, nitrogen mustards, nitroureas and aflatoxin which alkylate on the N7 position (see ref. 37 for review). It is possible that the low level of alkylation which is seen in these carcinogens may be related to the formation *in vivo* of Z-DNA structures. This is likely to increase their reactivity considerably. This subject can now be investigated using model sequences which readily form Z-DNA.

The G-C base pair forms part of the outer surface of Z-DNA; thus the C5 position of cytosine is likely to be more accessible there to an enzyme than in B-DNA. CpG sequences are relatively infrequent in eukaryotic DNA, but they are often highly methylated on cytosine C5, especially in inactive genes. As mentioned above, Z-DNA segments in B-DNA may be found as

units of CpG. Is it possible that these are sites for cytosine methylation? It has been speculated that the signal for methylation may be physical in nature³⁸, and an enzyme recognising a CpG Z-DNA segment may be the basis for this modification.

A full understanding of the molecular biology and chemistry of DNA requires that we understand not only the structure of B form DNA but also the structure of other conformations which the molecule can adopt. This is particularly true for those conformations which are significantly removed from the familiar right-handed double helical form of B-DNA. Z-DNA is the first such alternative conformation. It should serve as a stimulus in the search for other conformations of DNA which may provide greater depth and scope in our attempts to understand the molecular biology of DNA.

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Splicing of the late mRNAs of polyoma virus does not occur in the cytoplasm of the infected cell

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The three mRNAs that encode the capsid proteins of polyoma virus are produced by the excision of different sequences from continuous transcripts of the L strand of viral DNA. All three of the mRNAs have long half lives, and the larger species are not converted to the smaller ones to any measurable extent within the cytoplasm. Therefore the cytoplasmic proportions of late polyoma mRNAs are predetermined by splicing that is confined to the nucleus of the infected cell and which is complete by the time that mRNA is transported to the cytoplasm.

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CONCEPTS of gene expression in eukaryotes have undergone dramatic revision following the discovery that many of the RNAs synthesised in the nucleus comprise separate blocks of sequence within DNA which are brought together during transcript processing. The steps whereby the primary transcript of a gene is cleaved, certain 'silent' sequences (introns) excised and the remaining regions religated constitute what has been termed 'splicing'. These steps are important in the biosynthesis of mRNA¹⁻¹⁰, and also occur during the synthesis of ribosomal RNA (rRNA)^{11,12} and certain transfer RNAs¹³. The function of splicing in mRNA synthesis remains unclear. It has been proposed that it may have an evolutionary role in bringing together polypeptide domains with specific functions¹⁴. Furthermore, it may constitute an essential step in the synthesis of certain mRNAs. Recent experiments on deletion mutants of simian

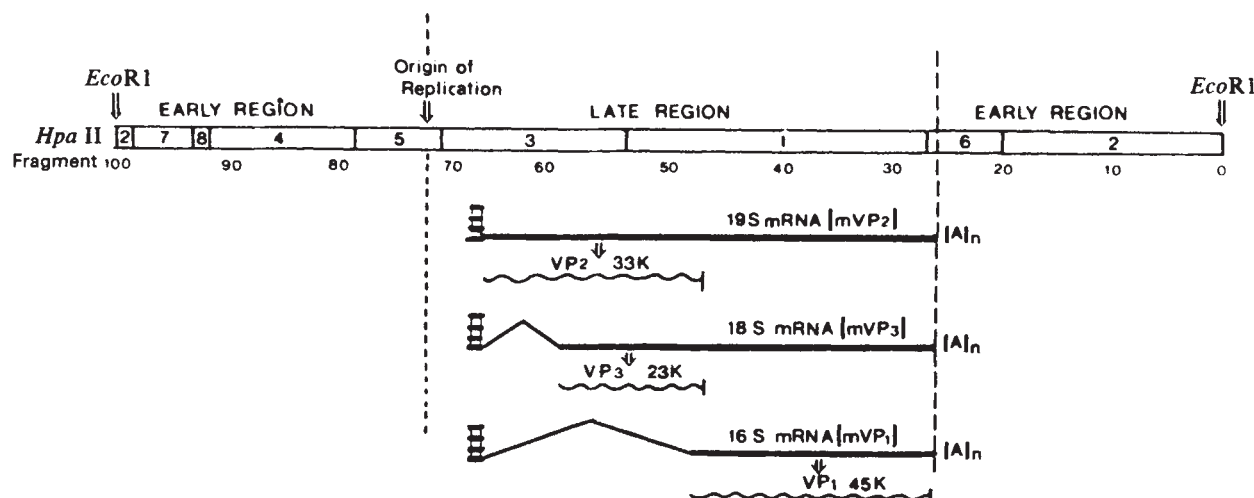


Fig. 1 The three late mRNA species of polyoma virus as they have been found to map^{21,22} relative to the restriction endonuclease *HpaII* cleavage map³⁵ of the viral DNA. The circular genome is represented here in linear form cleaved at the single restriction endonuclease *EcoRI* site on the DNA. Sequences conserved in each cytoplasmic mRNA are represented by thick lines, whilst those regions between the 5' leader and 3' body regions of each mRNA that are removed by splicing during mRNA synthesis are indicated by thin $\wedge$ -shaped lines. Waved lines denote capsid proteins and the regions on the mRNAs by which they are encoded²⁰.

virus 40 (SV40) defective in late mRNA production¹⁵ suggest that the splice junctions or the excised sequences are required for the appearance of the appropriate late mRNA in the cytoplasm. Removal of the sequence in the DNA that corresponds to the intron of the mRNA for SV40 VP1 protein completely abolishes appearance of this mRNA in the cytoplasm although the gene is still transcribed (G. Khoury, personal communication). Although splicing may be essential for the expression of certain genes, small amounts of apparently unspliced viral mRNA are, nevertheless, found in the cytoplasm of cells early in adenovirus infection¹⁶ and, as discussed later, also during papovavirus infections.

Many of the mRNAs of DNA tumour viruses have been found to fall into discrete groups, the members of each group having in common the sequences immediately preceding the poly(A) tract at their 3' termini. Some of the best characterised mRNA groups are the five groups of mRNA synthesised from the late transcription unit of adenovirus 2. Each group contains up to five individual mRNA species with common 3' terminal sequences adjacent to their poly(A) (refs 17–19). Another group of viral mRNAs sharing their 3' terminal sequences, studied in this investigation, is that produced late in polyoma virus infection. Such mRNA groups presumably owe their origin to alternative splicing processes that remove different regions of intervening sequence from the continuous transcripts of viral genomes that are detectable in the nuclei of cells infected with these viruses.

The late mRNAs of polyoma virus constitute more than 90% of the viral mRNA synthesised late in the lytic infection cycle^{20–23}. They comprise three distinct species that have been designated mVP2, mVP3 and mVP1, as they code for the VP2, VP3 and VP1 proteins of the virus capsid²⁰. These mRNAs have been mapped by Kamen *et al.*^{21,22} relative to the viral DNA, and their map positions and general structures are summarised in Fig. 1. The mRNAs share a 3' terminus at 25.3 map units on the DNA, with sequences extending to 66.5 (mVP2), 59.5 (mVP3) or 48.5 (mVP1) map units. These mRNA bodies are in turn covalently joined at their 5' ends (Fig. 1) to a small amplified leader sequence that maps at position 67 on the DNA²⁴. No splice has been detected after the leader sequence in mVP2, and available evidence indicates that it could involve the removal of only a few nucleotides if indeed it exists at all²¹. However, both mVP3 and mVP1 have a substantial part of the 67 to 25.3 map unit sequence removed during synthesis (Fig. 1). The related papovavirus SV40 produces only two late mRNAs, as it synthesises VP3 and VP2 by utilising two separate initiation sites on the same mRNA²⁵.

It is possible to envisage many ways by which the three mRNAs shown in Fig. 1 might be synthesised (through tran-

script processing within either the nucleus or the cytoplasm). For instance, synthesis could be restricted to the nucleus of the infected cell, each mRNA not undergoing further splicing after export to the cytoplasm. Alternatively only the largest species (mVP2) might be synthesised in the nucleus, whereupon the smaller mRNAs (mVP3, mVP1) may arise through a progressive cytoplasmic splicing of mVP2. A situation might also exist somewhere between these extremes. The experiments reported here favour the first alternative as they show that the relative proportions of the three mRNAs do not vary after export from the nucleus, and that cytoplasmic splicing of the larger mRNAs to yield the smaller species does not occur to any appreciable extent. It follows from this that the biosynthetic steps leading to the diversity of the polyoma late mRNAs occur exclusively within the nucleus.

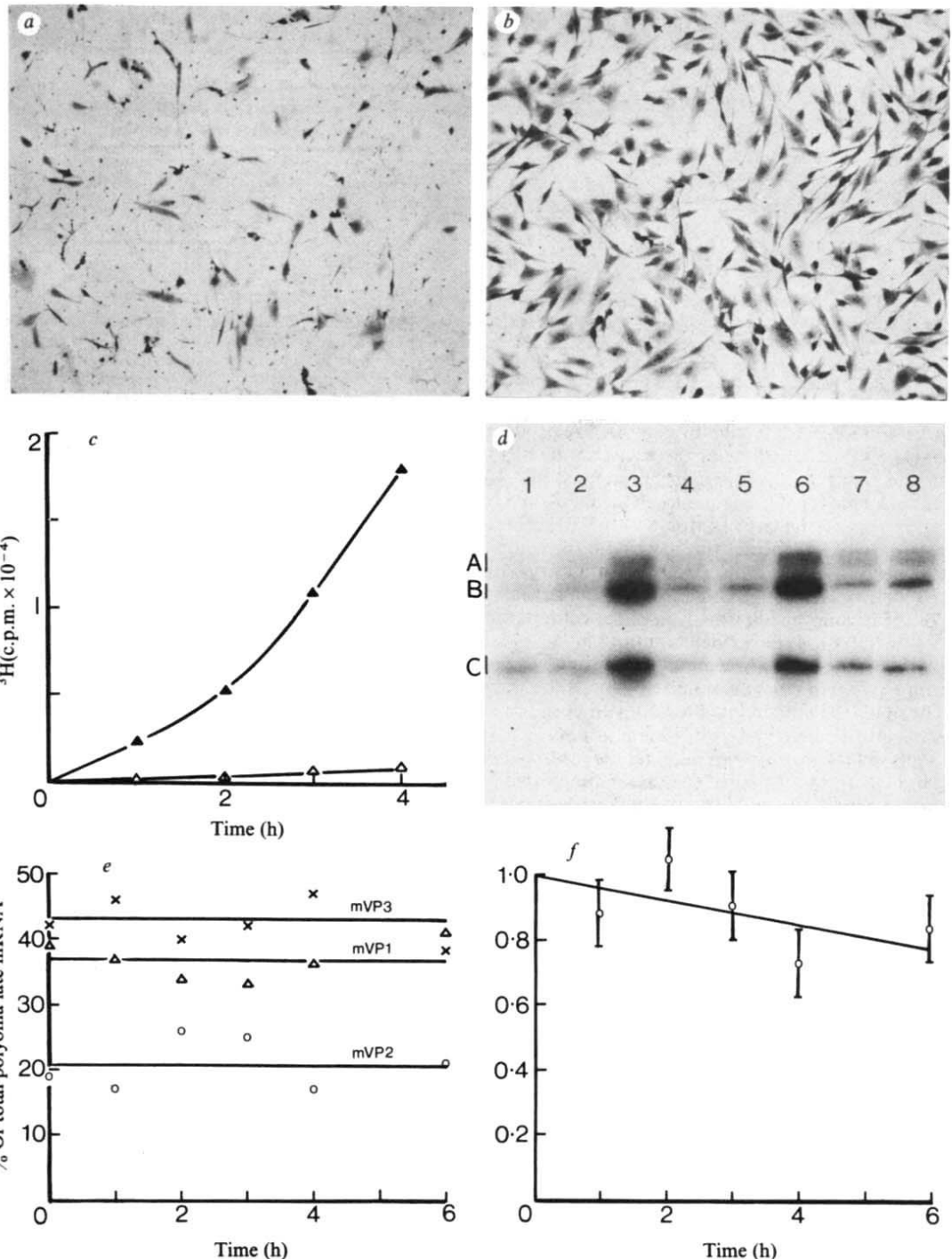
Although the three polyoma late mRNAs cannot be separated completely by gradient centrifugation²⁰ the 'S_i mapping' technique^{22,26} allows accurate measurement of their relative amounts in RNA samples, and of their labelling kinetics. We have used this technique to measure the decay of mVP2, mVP3 and mVP1 in enucleate cells, where mRNA synthesis cannot occur, and to observe the decay of these species in cells treated with actinomycin D at a concentration that completely inhibits mRNA synthesis. The technique has also been used to study radioisotope movement from each individual viral mRNA in a pulse-chase experiment.

The decay of polyoma late mRNA species in enucleate cells

Mouse 3T6 cells were enucleated 30 h after polyoma virus infection in cytochalasin B-containing medium, by a modification (Fig. 2) of the procedure described by Veomett *et al.*²⁷. A very substantial proportion of the cells in the flasks detach during the enucleation procedure. It was the cytoplasts remaining adhered that were used for the experiments, the yield per flask being rather variable. At least 98% of the cells not detached from the lower surface of the culture flasks during centrifugation in the presence of cytochalasin were found to be enucleated after staining (Fig. 2a). However, cells subjected to the enucleation procedure in the absence of cytochalasin retained their nuclei (Fig. 2b). In other experiments the cytochalasin treated cultures were found to retain not more than 4% of the ability to incorporate ³H-uridine into RNA exhibited by control cultures carried through the enucleation procedure in the absence of cytochalasin B (Fig. 2c). However, the cytoplasts maintained a constant rate of protein synthesis for more

Fig. 2 The stability of polyoma late mRNAs in enucleate cells. Subconfluent mouse 3T6 cells grown in Falcon flasks were infected at high multiplicity with A2 strain polyoma virus. They were then labelled with $10 \mu\text{Ci ml}^{-1}$ ^3H -uridine between 20 and 30 h after infection. Thirty hours postinfection the cells were treated at 37°C with medium containing $10 \mu\text{g ml}^{-1}$ cytochalasin B (Sigma), initially for 15 min in an incubator and then for 15 min whilst being centrifuged at 8,500 r.p.m. in a prewarmed Sorvall GSA rotor²⁷. During the latter stage the nuclei detach from the cells. The medium in the flasks was then immediately replaced with medium lacking cytochalasin and the cytoplasm incubated at 37°C for further periods ranging from 0 to 6 h. Total RNA was then prepared from enucleate cells incubated for various times following enucleation²³ and the poly(A)⁺ component of these RNA samples prepared by oligo dT cellulose chromatography³⁶.

a, Enucleate infected cells, fixed and stained 1 h after removal of cytochalasin B. **b**, Virus-infected cells carried through the enucleation procedure in parallel with those in (a) except without the cytochalasin treatment. Fewer cells have detached during centrifugation and those remaining have not lost their nuclei. **c**, Control experiment to measure the time course of incorporation of ^3H -uridine into trichloroacetic acid-insoluble material immediately after the enucleation procedure had been carried out either in the presence (Δ) or in the absence ($\blacktriangle$) of $10 \mu\text{g ml}^{-1}$ cytochalasin B. Label was added at the point in the procedure where the cytochalasin is removed. **d**, Analysis by S_1 mapping of the content of late viral mRNA species in enucleate cells at various times after enucleation. Bands A, B and C correspond to the DNA protected from S_1 nuclease digestion through hybrid formation to the mVP2, mVP3 and mVP1 mRNA species respectively^{21,22}. The samples analysed in the hybridisations, corresponding to the numbered tracks in (d), were: 1, cytoplasmic poly(A)⁺ RNA from cells carried through the enucleation procedure in the absence of cytochalasin; 2, 3, 4, 5, 6 and 7, poly(A)⁺ RNA from cytoplasm incubated for 0, 1, 2, 3, 4 and 6 h, respectively after enucleation. 8, The same sample as track 2, except hybridisation was performed using only one third (50 μg) the amount of DNA used in the other annealings as a check that the other hybridisations were in DNA excess conditions. **e**, The relative proportions of each late polyoma mRNA in cytoplasm at different times following enucleation, obtained by quantitation of the tracks in (d) and expressed, to an accuracy of $\pm 6\%$, as a percentage of the total viral mRNA in enucleate cells. This is expressed as a fraction of the relative proportions of these RNAs in cytoplasm immediately after enucleation, and was calculated from the data in **e** and the ^3H label remaining as 18S and 28S rRNAs in the poly(A)⁺ component of the same RNA samples, measured after fractionation of these species on agarose gels.



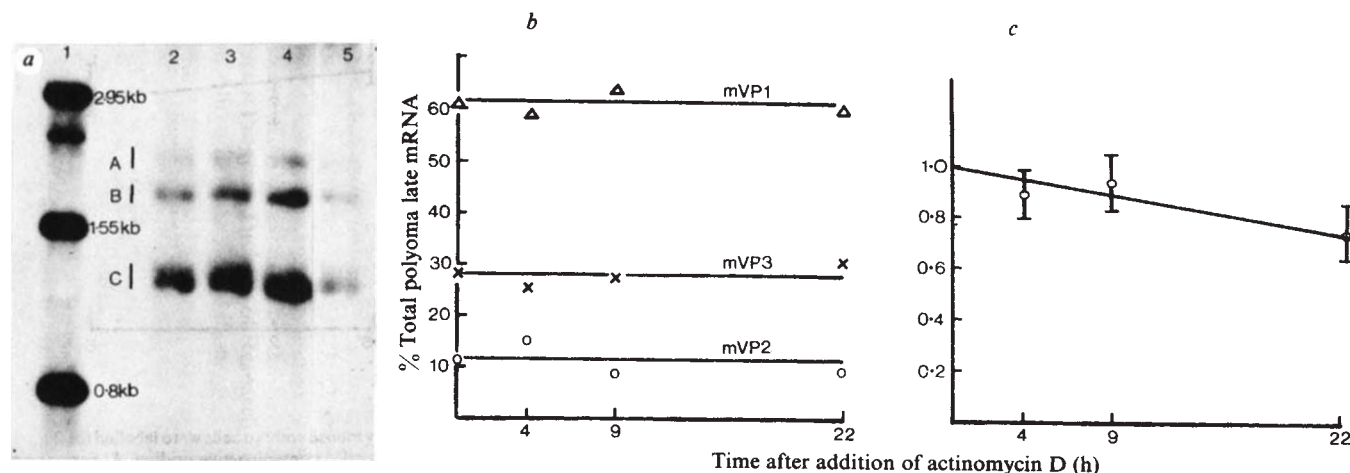


Fig. 3 The stability of polyoma late mRNAs following actinomycin D treatment. Confluent secondary mouse embryo cells were labelled with $10 \mu\text{Ci ml}^{-1}$ ^3H -uridine between 10 and 30 h after high multiplicity infection with polyoma virus. Actinomycin D ($5 \mu\text{g ml}^{-1}$) was then added to the cells, and batches of approximately 5×10^7 cells collected 0, 2, 5 and 22 h following addition of the drug. Cytoplasmic RNA was then prepared from these cells²³, poly(A) selected³⁶ and 10- μg aliquots of each poly(A)⁺ RNA annealed to an excess (100 μg) of restriction endonuclease *Eco*R1-cleaved DNA. Hybridisation, *S*₁ nuclease digestion, agarose gel fractionation of protected hybrids and hybrid detection were as described in Fig. 2. *a*, *S*₁ mapping analysis of the polyoma mRNA content of each cytoplasmic RNA sample. Track 1 contained 20 ng molecular weight marker restriction endonuclease SST digestion fragments of polyoma DNA. Tracks 2, 3, 4 and 5 show polyoma DNA protected from *S*₁ nuclease digestion through hybrid formation with cytoplasmic viral mRNAs of cells treated for 0, 4, 9 and 22 h respectively with actinomycin D. Bands A, B and C, as in Fig. 2, reflect the levels of mVP2, mVP3 and mVP1. *b*, The relative proportions of each of the three polyoma late mRNAs, expressed as a fraction of the total, at times after actinomycin addition. These were determined to an accuracy of $\pm 4\%$ by quantitation of individual tracks in (*a*), as described in Fig. 2. *c*, The stability of the total viral late mRNA relative to the stability of rRNA. These values were calculated from the data in (*b*) and measurements of the degree of ^3H -uridine labelling of 18S and 28S rRNA at each time point and are expressed as a fraction of the relative proportion of viral mRNA to total rRNA at the time of actinomycin addition.

than 6 h, which only declined dramatically 12–15 h after enucleation (not shown). For analysis of their viral mRNAs, cytoplasmic RNA was refed with fresh medium lacking cytochalasin immediately after enucleation and incubated for further periods. Then at various times 0–6 h after enucleation two flasks were removed from the incubator and RNA was prepared from the cytoplasmic RNA remaining adhered to their lower surfaces. The content of polyoma mRNAs in these RNA samples was analysed later, to measure the relative proportions of the three viral late mRNAs in cytoplasmic RNA at different times after enucleation. Initially the poly(A)⁺ components of these RNA samples were hybridised to an excess (150 μg) of linear restriction endonuclease *Eco*R1-cleaved polyoma DNA in conditions such that RNA–DNA annealing is possible, but DNA–DNA annealing does not occur^{21,22,26}. The annealed samples were then digested with single strand specific nuclease *S*₁ and the protected hybrids alkali denatured^{22,26} before fractionation on a 2% agarose gel. DNA was then transferred from the gel to a sheet of nitrocellulose by blotting²⁸ and the filters annealed to a ^{32}P -labelled polyoma DNA probe synthesised *in vitro* by DNA polymerase-catalysed nick-translation of viral DNA^{21,22}. Blots were washed and autoradiographed (Fig. 2*d*). The ratio of mVP2, mVP3 and mVP1 in the original RNA samples was measured from the amount of DNA that each mRNA protected from *S*₁ nuclease digestion through hybrid formation, present as the appropriate band on the blot (Fig. 2*d*). The variation in intensity of the tracks in Fig. 2*d* reflects the fact that the RNA of each flask of cytoplasmic RNA was hybridised to a vast excess of viral DNA without correction for the variable yield of enucleate cells.

The amount of each of the late polyoma mRNAs in enucleate cells at various times after enucleation is expressed in Fig. 2*e* as a percentage of the total viral mRNA present. The data indicate that there was no difference between the rates that mVP2, mVP3 and mVP1 were being degraded in the enucleate cells. They also show that conversion of the larger late polyoma mRNAs to the smaller ones through cytoplasmic splicing was not occurring to any measurable extent. When the amount of polyoma mRNA, obtained from the blot hybridisation, was calculated relative to the ^3H -uridine label present as rRNA in each RNA sample (Fig. 2*f*), it was evident that all three polyoma late mRNAs have long (>10 h) half lives in the enucleate cells. Ribosomal RNA is a convenient stable RNA against which to

relate the stability of an mRNA. Also it is necessary to use this latter correction to estimate RNA half lives, as a large proportion of the infected cells detach from the flask during enucleation the resultant variable yield of enucleate cells in each flask being reflected in the variable yield of viral mRNA (reflected in Fig. 2*d*). It is also apparent from Fig. 2*d*, *e* that the ratio of the three polyoma late mRNAs in the enucleate cells is rather different from that found in normal cells late in infection, there being relatively more mVP2 and mVP3 and correspondingly less mVP1. Although we have not definitely established why this is so, cells late in polyoma infection attach less firmly to their substratum and the enucleation procedure probably selects preferentially those cells least advanced in the lytic infection cycle. Also we have observed that the relative molar ratios of mVP2:mVP3:mVP1 in the cytoplasm of cells late in infection can vary as widely as 1:1:2 to 1:2:7 between different cultures, and that there is generally a relative increase in the amount of mVP1 at later times during infection.

The decay of polyoma late mRNAs after inhibition of mRNA synthesis by actinomycin D

To investigate further whether the polyoma late mRNAs undergo cytoplasmic interconversion or if they each constitute long-lived species, we determined the ratio of mVP2, mVP3 and mVP1 in the cytoplasm of infected cells at different times following addition of $5 \mu\text{g ml}^{-1}$ actinomycin D to the medium. At this concentration actinomycin D completely inhibits mRNA synthesis and greatly interferes with the nuclear maturation of mRNA precursors²⁹. Cells late in polyoma infection were prelabeled with ^3H -uridine and then treated with actinomycin D, as described in Fig. 3. At various times after addition of the drug, cytoplasmic RNA was prepared from a fraction of the culture. The content of polyoma late mRNAs in these RNA samples was then determined (Fig. 3*a*, *b*) as detailed in Figs 2 and 3 legends.

Following actinomycin treatment the ratio of the three polyoma late mRNAs in the cytoplasm of infected cells does not undergo discernible change, even during prolonged incubation (Fig. 3*a*, *b*). These mRNAs also have long half lives, as is apparent when their stability is compared to that of rRNA (Fig.

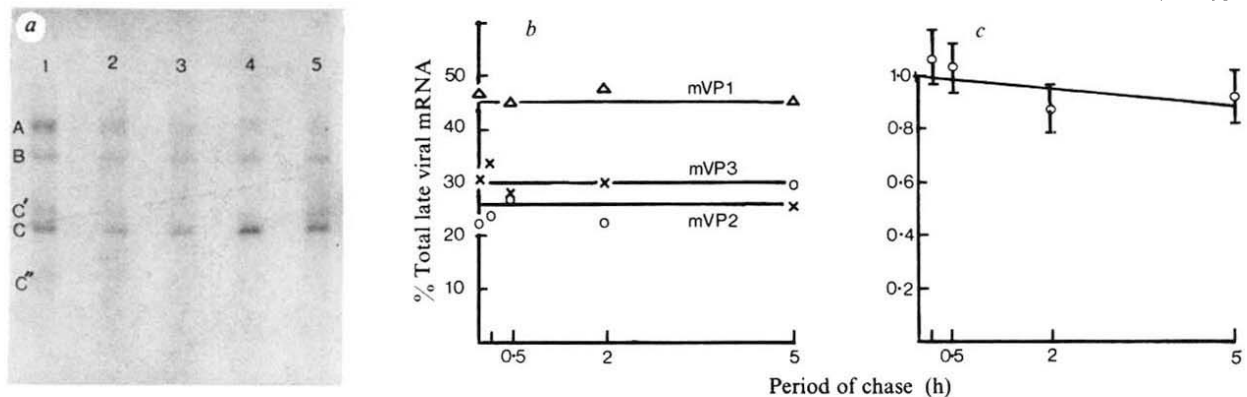


Fig. 4 Stability of ^3H -uridine pulse-labelled polyoma mRNAs over a 5-h chase period. Confluent virus-infected secondary mouse embryo cells were labelled for 2 h in medium containing $40 \mu\text{Ci ml}^{-1}$ ^3H -uridine. The label was then removed and replaced with medium containing $10 \mu\text{g ml}^{-1}$ nonradioactive uridine. At various times of chase from 0 to 5 h a sample of 5×10^7 cells was collected, and cytoplasmic RNA prepared²³. The poly(A)⁺ fractions of these RNA samples were then annealed to an excess (0.5 μg) of restriction endonuclease *Eco*R1-cleaved polyoma DNA. Hybridisation and S_1 nuclease digestion were as described in Fig. 2 legend. Nuclease-resistant hybrids were fractionated undenatured on a 2% agarose gel. The gel was soaked for 10 min in 5% trichloroacetic acid, impregnated with 2,5-diphenyloxazole (PPO) and ^3H detected initially by fluorography³⁷. An autoradiograph of the gel is shown in (a). In addition to the major bands A, B and C resulting from mVP2, mVP3 and mVP1 two additional weaker bands (C', C'') originating from mRNAs synthesised from the early region (Fig. 1) of the polyoma genome²² were also detected in this particular experiment. Tracks 1, 2, 3, 4 and 5 are from poly(A)⁺ cytoplasmic RNAs isolated after 0, 10, 30 min, 2 h and 5 h chase periods respectively. Each track was subsequently sliced to determine the amount of label present as individual late viral mRNAs. As in Figs 2e and 3b these values in (b) are expressed as a fraction of the total polyoma late mRNA at each time point ($\pm 4\%$). The stability of total viral late mRNA is also expressed in (c), as in Figs 2f and 3c, relative to the stability of rRNA, the values being given as a fraction of this ratio at the beginning of the chase.

Fig. 3c). The amount of nuclear viral mRNA precursor present at the time of actinomycin addition is small relative to the total amount of viral mRNA in the cytoplasm and its processing would increase the total amount of the latter by less than 5% (unpublished results). Furthermore the processing of these precursors is probably strongly inhibited by the actinomycin²⁹. A more serious difficulty in the interpretation of the experiment in Fig. 3 is the possibility that actinomycin might be inhibiting a cytoplasmic processing of the larger late polyoma mRNAs to the smaller species. Likewise it could be argued that a similar processing of these three mRNAs is being prevented in enucleate cells through the absence of a labile factor normally contributed by the nucleus. We therefore designed a pulse-chase experiment to demonstrate the absence of such an interconversion in nucleate infected cells in the absence of RNA synthesis inhibitors.

The chasing of radioactivity in labelled late polyoma mRNAs

Another approach to determining whether the late polyoma mRNAs have different half lives or undergo cytoplasmic interconversion is to analyse the kinetics with which radioactivity is chased from these species after a pulse label. In the experiment described in the legend to Fig. 4 infected cells were labelled for 2 h with ^3H -uridine so as to obtain a fairly high degree of labelling of viral mRNA. The isotope was then removed and medium containing nonradioactive uridine substituted. At various times from 0 to 5 h a portion of the cells was collected, cytoplasmic RNA prepared, and the polyadenylated fraction of this RNA isolated by oligo(dT)-cellulose chromatography as for the earlier experiments. These poly(A)⁺ fractions were then annealed to an excess of viral DNA and the annealings digested with nuclease S_1 , whereupon the protected hybrids were fractionated undenatured on an agarose gel (Fig. 4a). The degree of labelling of each of the three mRNA species after different times of chase (Fig. 4b) again showed no differential stability, the labelling ratio being preserved throughout the entire 5-h chase period. The labelling of these mRNAs relative to that of rRNA was also consistent with the long (>10 h) half lives suggested by the other experiments (Fig. 4c). The processing of nuclear viral mRNA precursors labelled before the start of the chase does not have a large effect on the data in Fig. 4. We have shown in other experiments (unpublished) that after labelling infected cells for 2 h with ^3H -uridine in these conditions 30–50% of the label

present as viral RNA is present in the nucleus. However, less than 10% of this fraction is exported to the cytoplasm as mRNA, this component contributing no more than 3–5% of the total label as viral mRNA during the first 1–2 h of the chase period in Fig. 4. Reasonable chase conditions were successfully obtained in this experiment as the specific activity of cytoplasmic total tRNA did not rise after the second time point. Measurements of tRNA labelling constitute a good control for the establishment of satisfactory chase conditions since tRNA is very rapidly exported from the nucleus after its synthesis²⁹.

Conclusion

These results indicate that the late mRNAs of polyoma virus have long half lives and that polyoma mVP2 is not to any significant degree spliced to either mVP3 or mVP1 in the cytoplasm of virus-infected cells. It follows from this that the differences in splicing pattern responsible for the diversity of these species must arise before the three mRNAs enter the cytoplasm, either within the nucleus or at the latest during mRNA transport to the cytoplasm. Studies of the processing of nuclear viral transcripts will eventually reveal whether in the nucleus mVP2 is the immediate precursor to mVP3 and mVP1, or if predestination occurs much earlier in transcript maturation, each species thereafter having a separate biosynthetic pathway. Applying the tentative rules for the recognition sequences of splicing enzymes on RNA³⁰ to the sequence of polyoma DNA³¹, it is found that splicing mVP2 to mVP3 should preclude a further splice from mVP3 to mVP1. On this basis alone mVP2 might be the nuclear precursor of mVP3 and mVP1, yet mVP1 synthesis should not proceed through mVP3.

Although there is some evidence that splicing occurs during the processing of certain transcripts of the mitochondrial genome¹² maturation of these transcripts is probably confined to the mitochondrion. Apart from this there have been no clear demonstrations that RNA splicing occurs in the cytoplasm of eukaryotic cells. There have also been no reports of the occurrence of such processes during the infective cycle of poxviruses, a class of DNA viruses that replicate within the cytoplasm of their host cells³³. Also the nuclear processing intermediates that constitute cellular mRNA precursors³² and viral mRNA precursors²¹ are not found in appreciable amounts in cytoplasmic extracts. However, our results with the late mRNAs of polyoma virus are at variance with those of an earlier study of a similar kind. Aloni *et al.*³⁴ investigated the stability of the late SV40

mRNAs and showed that the larger mRNA species was more rapidly labelled in the cytoplasm following addition of radioisotope. This result is also obtained on labelling the late mRNAs of polyoma virus *in vivo* for a continuous period of 1–2 h (unpublished results) and can be variously interpreted as either reflecting a difference in times of mRNA transport from nucleus to cytoplasm or a cytoplasmic conversion of the larger mRNAs to the smaller ones. Aloni *et al.*³⁴ concluded from sucrose gradient analysis of the viral RNA of enucleate SV40-infected

cells that the latter process was taking place. However, the half lives (2–8 h) that they measured for the viral mRNAs of the enucleate cells are considerably shorter than in the present study and there have been no experiments described to verify these half-lives under more physiological conditions.

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letters

X-ray and optical variability in 2A1052+606 —a new RS CVn-type system

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The RS CVn binaries have been found to be a new class of soft X-ray sources from the HEAO 1 low energy sky survey¹. We discuss here a star with RS CVn-type characteristics that was discovered as a result of an accurate X-ray location². We have detected this source at energies <1 keV and present evidence for variability on time scales ~1 day. Subsequent spectrophotometry revealed variable H α and possibly Ca II H, K emission which strongly supports its classification as an RS CVn system.

The most extensive new class of soft X-ray sources discovered by the HEAO 1 low energy detectors (LEDs) are the RS CVn binaries¹. Their characteristics have been reviewed by Hall³ and their X-ray properties have been summarised by Walter *et al.*⁴. Most of these binaries, which usually consist of a K0 IV and a ~G main sequence star³, have X-ray luminosities in the range 10^{30} – 10^{32} erg s⁻¹ and temperatures ~ 10^7 K (refs 1, 5). One such system, HR1099, has already been shown to be variable at hard X-ray energies⁶. The high latitude X-ray source 2A1052+606

(ref. 7) has recently been identified⁸ with a bright star (SAO 015338=BD+61°1211; $V \sim 9$) as the result of an accurate X-ray location² from the HEAO 1 modulation collimator experiment. This star is suspected of being an RS CVn-type given its variable H α emission^{2,13} and 7.5 d binary period⁹. Here we use data from LED 1 of the HEAO A2 experiment¹⁰ to study the soft X-ray nature of 2A1052+606.

During 5–6 November 1977 a strong soft X-ray source at a position consistent with 2A1052+606 and SAO 015338 was observed. The X-ray light curve (Fig. 1) shows that the source underwent a strong soft X-ray flare. This variability is clearly demonstrated when the data is tested against a model of constant intensity, yielding a χ^2 of 3.7 per degree of freedom. Assuming the identification, the flare reached a maximum X-ray luminosity of $1.7 \pm 0.17 \times 10^{32}$ erg s⁻¹ (0.2–2.8 keV). The rise time of the flare was short (~2 h) and the decay was considerably slower; the total X-ray energy in the flare was $\sim 4 \times 10^{37}$ erg. X-ray observations 6 months later give an upper limit of 6×10^{31} erg s⁻¹ for the source intensity. The weak residual intensity observed after the flare is probably not associated with the quiescent source. The hard Ariel 5 source, 2A1052+606 (ref. 7), has a mean, steady luminosity of $\sim 3 \times 10^{31}$ erg s⁻¹ in the 2–10 keV band.

The spectrum obtained during the flare is indistinguishable from that of UX Ari¹ with a temperature of $T \sim 10^7$ K, and shows no evidence of Fe XVII–XVIII line emission. There is no detectable change in the spectrum during the decay (7–9 November 1977). The X-ray characteristics are similar to those of the more active RS CVn systems, notably to HR1099, which

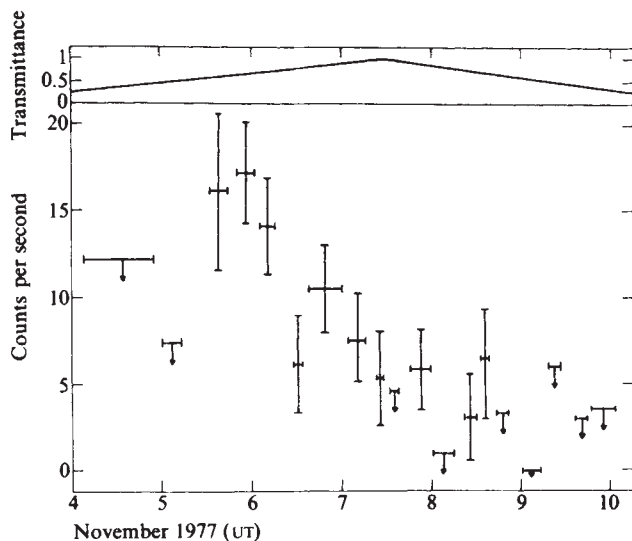


Fig. 1 Aspect corrected 0.2-2.8 keV light curve of 2A1052+606 from HEAO 1 LED 1 observations during November 1977. The time of peak source transit was day 311 assuming the identification with SAO 015338; the variability is clear. The upper plot shows the collimator transmittance to a source at the position of SAO 015338.

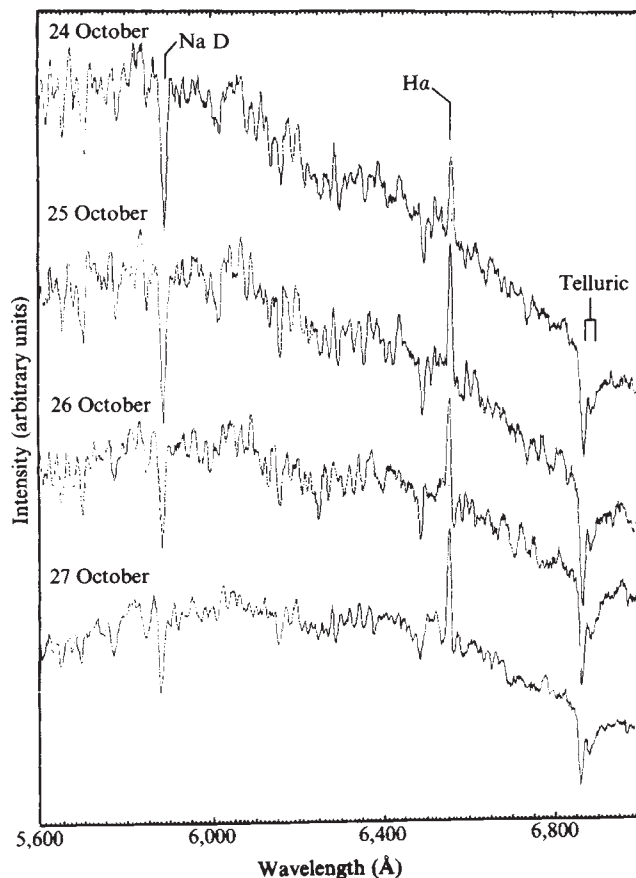


Fig. 2 Section of Lick 3 m ITS scans on 24-27 October 1978 which covers the region of H α . The variability by a factor ≥ 2 from night to night is clearly evident. The Na D absorption is contaminated by varying amounts of night sky emission.

flares⁶ in variability, and UX Ari in spectrum. The upper limit to the quiescent luminosity is consistent with that observed in other RS CVn systems¹¹ and the Ariel 5 intensity of this source. The different kinds of variability observed in RS CVn systems (HR1099, UX Ari, Capella) and their implications in the coronal loop model are discussed elsewhere¹¹.

After the X-ray observations were completed we observed SAO 015338 optically to search for spectroscopic variability at Ca II H, K and H α . Spectrophotometric data were taken with the Lick Observatory 3 m Shane telescope and image tube scanner¹² during a four night run in October 1978. Figure 2 shows the nightly mean spectra around H α plotted in arbitrary intensity units (poor atmospheric observing conditions on three out of four nights precluded accurate flux calibration during the time of these observations). The H α variability is clearly visible and there is weaker evidence of a variable amount of 'filled-in' Ca II H, K. The Na D lines are contaminated by varying amounts of night sky emission. The H α variability is one of the classical indications of RS CVn-type activity superimposed on an otherwise normal late-type stellar spectrum³. Our data strongly suggest that this star should be added to the list of X-ray emitting RS CVn systems. In addition, long time-base optical photometry has recently¹⁴ yielded evidence of an unusually large amplitude (0.3 mag) photometric wave that is superimposed on the binary period. Subsequent observations during the next few years should show that this wave shifts gradually with phase³.

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X-ray emission from γ -ray sources in the galactic anticentre region

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An X-ray survey of three high-energy γ -ray sources in the galactic anticentre region has been conducted using the large area sky survey (LASS) instrument on HEAO 1. The sensitivity of this survey ≈ 0.5 Uhuru flux units (UFU) is the highest yet reported. Although we do not confirm the recently published detection¹ of an X-ray source associated with γ 195+5 (CG195+4); we report the detection of two, and possibly three, other X-ray sources found in its vicinity. Other than the supernova remnant, IC443, no other discrete X-ray sources have been detected near CG189+1, but there is evidence for diffuse

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emission or emission from unresolved sources in its vicinity. Three new X-ray sources have been discovered within a few degrees of CG176-7, but none overlaps the γ -ray error box. These results demonstrate that γ -ray sources like CG195+4 and CG176-7 have emission characteristics significantly different from the Crab pulsar, but may be similar to the Vela pulsar. Alternatively, the lack of a detectable X-ray flux suggests that the γ -ray emission arises from cosmic-ray interactions in dense interstellar clouds. An interesting feature of the new X-ray sources detected in this survey is that four of them may be associated with early-type stars which exhibit emission lines.

Following the detection of 13 localised sources of high-energy γ -radiation⁵, attempts have been made to correlate them with a variety of astrophysical objects. These include X-ray sources³⁻⁶, optical counterparts⁷, H II regions⁸, supernova remnants⁹⁻¹¹, highly variable galactic radio sources¹², pulsars and other compact objects¹³⁻¹⁴, quasars¹⁵, and large scale galactic features¹⁶. To date 24 localised regions of γ -ray emission have been detected by COS B (ref. 17). Four of the sources are known from their time signatures to be associated with pulsars (PSR0531+21, PSR0740+28, PSR0833-45, and PSR1822-09). Identifications of the remaining γ -ray sources with other astrophysical objects remain inconclusive. Positions of two of the γ -ray sources, CG135+1 and CG291+65, are consistent with the quasars, 0241+622 (ref. 15) and 3C273 (ref. 18), respectively. However, the highly variable galactic radio source GT0236+61 (ref. 12), the diffuse nebula IC1805 (ref. 10) and the H II region W3 (IC1795)⁸ are also consistent with the position of CG135+1.

HEAO 1 has made it possible to investigate regions of localised γ -ray emission for X-ray sources a factor of 5 or more weaker than have previously been catalogued¹⁹. Results have already been reported from a survey of the γ -ray sources CG135+1, CG312-1, CG327-0 and CG333+0 using data from the NRL LASS experiment⁶. A weak X-ray source, H0235+60, with an intensity of 3.5×10^{-3} counts $\text{cm}^{-2} \text{s}^{-1}$ in the 1-20 keV range (~ 0.6 UFU, for a Crablike spectrum) was discovered whose position is consistent with the location of CG135+1 and also possibly GT0236+61 (ref. 12). No resolved discrete sources were found near CG312-1, but enhanced galactic emission from unresolved sources or diffuse radiation was detected. The survey of the region near CG327-0 and CG333+0, as might be expected due to its proximity to the galactic centre region, revealed at least three new X-ray sources. The position of H1613-51 is consistent with the position of CG333+0; the other sources lie $\geq 1^\circ$ away from the γ -ray

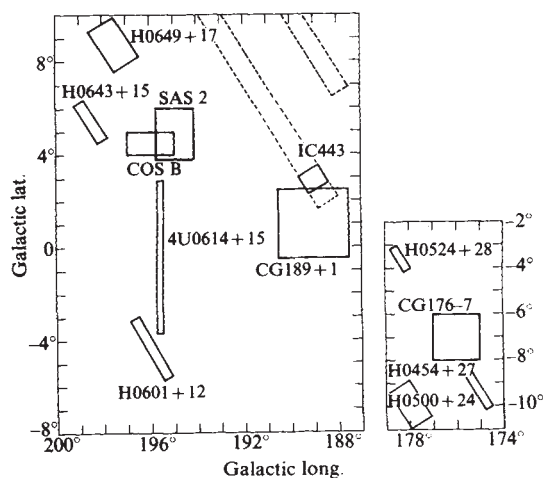


Fig. 1 Locations of three γ -ray sources near the galactic anticentre and positions (90% confidence error boxes) of newly discovered X-ray sources in their vicinity. The error box of IC443 reflects its extended region of emission. The dashed lines contain regions in which enhanced X-ray emission due to unresolved multiple sources or diffuse radiation has been observed. Both the SAS 2 and COS B positions for CG195+4 are shown.

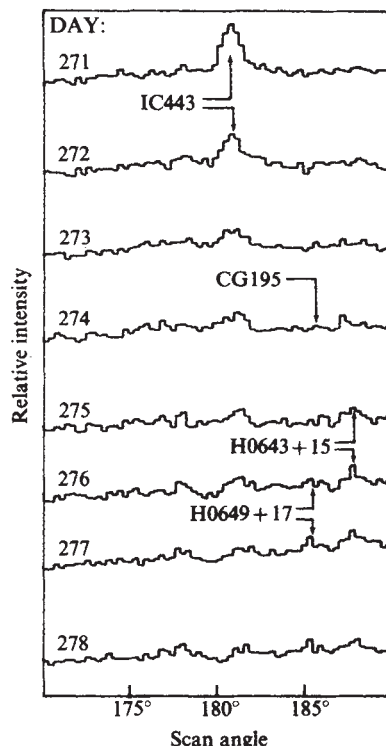


Fig. 2 Two-day sums of scans through the regions near CG189+1 and CG195+4 beginning on the day in 1977 specified. The arrows identify the scan angles at which specific sources are observed. The 2-day sum in which the intensity from an X-ray source at CG195+4 would be a maximum is also identified.

positions. As shown in Fig. 3 of ref. 6, the optically visible⁷ supernova remnant RCW 103 is centred in the error box of H1613-51 and the radio supernova remnant MSH16-52 lies near its edge. Also, the most luminous H II region measured in the 1-25 μm band²⁰ lies near the centre of the γ -ray position.

The HEAO 1 LASS X-ray observations in the vicinity of three unidentified γ -ray sources, CG176-7, CG189+1, and CG195+4 located in the galactic anticentre region were made in September and October 1977. Four 1,600 cm^2 detectors were used to observe CG176-7, while two detectors were used to observe CG189+1 and CG195+4. Each module had a field of view of $1^\circ \times 4^\circ$ FWHM, with the 1° aperture in the scan direction. The experiment scans the great circle perpendicular to the satellite spin axis every 30 min. Therefore, the sources were observable for ~ 10 s during each scan with the exception of times when they were occulted by the Earth or when the satellite passed through high radiation zones. Because the spin axis follows the Sun, the sources are visible for up to 8 days every 6 months.

The results of this investigation are summarised in Fig. 1. The region near CG176-7 exhibited extended emission; however, three new discrete X-ray sources have been resolved. Information on these sources is summarised in Table 1 which gives the position of the centre of the error box, the area of the error box, the source intensity in the 1-20 keV range, and possible identifications. For comparison with other instruments 5×10^{-3} counts $\text{cm}^{-2} \text{s}^{-1} \approx 1$ UFU for a spectrum similar to the Crab.

Figure 2 shows summations of two days of scans in the vicinity of the γ -ray sources CG189+1 and CG195+4 starting on the day indicated. The X-ray source associated with the supernova remnant IC443 is seen leaving the 4° field of view of the detectors. It was centred in the field of view on day 269; the γ -ray source CG189+1 was centred on day 268. Unfortunately, due to instrumental problems, we have no data for most of days 268-269. This made it difficult to resolve additional X-ray sources near CG189+1. As Fig. 2 shows, there is evidence for

an additional source or sources near IC443. This extended region of emission near IC443 is shown by the dashed lines in Fig. 1 (a second emission region is also shown in the upper right corner). Part of this extended emission is due to the extent of IC443 (ref. 21), ($\sim 0.7^\circ$), but part may also be due to an additional weak source consistent with the position of CG189+1. A point summation analysis of data from Uhuru⁴ revealed evidence for an X-ray source near CG189+1, distinct from IC443. It was observed at the 2.7σ confidence level with an intensity of ~ 3 UFU. Our observations indicate that any X-ray source at the reported position has an intensity which is < 0.5 UFU. If the new source associated with CG189+1 is real, it must be highly variable.

The γ -ray source identified in the COS B catalogue as CG195-4 was first detected by SAS 2 (ref. 22). Both the SAS 2 and COS B positions are shown in Fig. 1. Recently the A2 experiment on HEAO 1 has provided evidence for a weak X-ray source lying almost entirely within the SAS 2 error box¹. The detection was based on observations in 1977 October and 1978 April with a combined statistical significance of only 3.1σ . A point summation analysis of data from Uhuru⁴ also provided marginal evidence for X-ray emission near that location. Our results are based on the LASS observation during 1977 October. The arrow in Fig. 2 identifies the scan in which an X-ray source associated with CG195+4 would have been centred within our detectors' field of view. A very weak source appears at the expected scan angle, but it peaks about 3 days later than expected for the γ -ray source. We treat this source, H0649+17, as a marginal detection because it was observed for only 3 days, with a maximum statistical significance of 4σ on a single day. Its intensity is ~ 0.5 UFU. A much stronger source (~ 1 UFU) H0643+15, is displayed in Figs 1 and 2 and was observed above the 5σ level on each of 3 days. It also falls well away from the position of the γ -ray source. 4U0614+15 (~ 5.5 UFU) also lies near the γ -ray position, but has a large uncertainty in galactic latitude. We observed a source designated as H0601+12 which lies just near the edge of the Uhuru position. Its intensity is ~ 0.7 UFU, which is significantly weaker than 4U0614+15, but it may very well be the same source. Further information on the X-ray sources near CG195+4 is given in Table 1.

We, therefore, do not confirm the reported detection¹ of an X-ray source associated with $\gamma 195+5$. Some discussion concerning this disagreement is necessary. As noted above and shown in Fig. 2, the source which we designate as H0649+17 appeared at the scan angle expected for $\gamma 195+5$ but peaked in

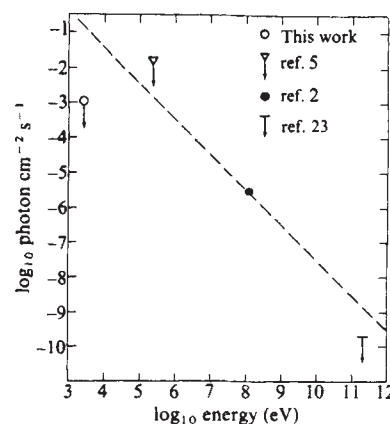


Fig. 3 Integral photon spectrum of CG195+4. The dashed line represents an $N(>E) \propto E^{-1}$ spectrum normalised to the observed intensity > 100 MeV.

intensity ~ 3 days too late. It is possible that this was the source detected by the A2 experiment but, that due to their marginal statistics, the position they obtained was in error. But this brings up a puzzling point, the HEAO A2 survey covered a $10^\circ \times 5^\circ$ region about $\gamma 195+5$. Why did they fail to detect the relatively strong source H0643+15 found in our survey? We also do not believe that our failure to detect the A2 source is due to source variability because: (1) our observations were made during the same October 1977 viewing period as one of the A2 periods, and (2) the intensity of the source as measured by A2 seemed to be about the same for both periods.

We obtain a 2σ upper limit of 3×10^{-3} counts $\text{cm}^{-2} \text{s}^{-1}$ (1–20 keV; ~ 0.6 UFU for a spectrum similar to the Crab) for the intensity of an X-ray source at $\gamma 195+5$. This compares with the A2 measured energy flux of $1.5 \pm 0.5 \times 10^{-11}$ erg $\text{cm}^{-2} \text{s}^{-1}$ (2–6 keV) or 1.1 UFU. Because our instrument response extends to lower energies than the A2 data, it is relatively even more sensitive to this 'new' source whose spectrum appears to be softer than the Crab. Thus our 2σ upper limit is at least a factor of two below the A2 measured intensity.

To learn something about the emission mechanism of $\gamma 195+5$ (CG195+4), we compare our upper limit to the X-ray intensity with measurements at other energies in Fig. 3. The only positive detections of the source are in the 100-MeV region^{2,22}. The upper limit set in the 260–1,230 keV range was obtained from the Imperial College scintillation telescope on Ariel 5 (ref. 5). Emission from CG195+4 was also not detected in a recent investigation of very high energy γ -ray emission using the atmospheric Cerenkov technique²³ and an upper limit was placed for energies $> 2 \times 10^{11}$ eV. For comparison with the upper limits, we show by the dashed line an assumed integral power law spectrum ($\propto E^{-1}$) which is harder than that observed for the Crab (as is the case for the γ -ray emission from CG195+4) and which is normalised to the observed 100-MeV intensity. The LASS limit in the X-ray band falls two orders of magnitude below this extrapolation. A similar conclusion is reached with respect to CG176-7. These γ -ray sources, therefore, exhibit spectra that are similar to that of the Vela pulsar, for which to date no pulsed X-ray emission has been detected²⁷. An alternative explanation for the failure to detect X-ray emission from these sources is that the γ rays are produced in cosmic-ray collisions with interstellar clouds such as has been suggested in ref. 11.

None of the new X-ray sources detected by LASS is consistent with the positions of the γ -ray sources. However, we note that the γ -ray positions and their uncertainties are not well known²⁶. It cannot be entirely ruled out, therefore, that one of these nearby X-ray sources may be a counterpart.

There is also an interesting association of four of these new X-ray sources with early type stars which exhibit emission lines. We estimate that the probability of randomly obtaining this

Table 1 New X-ray sources

Source	Position (1950) RA Dec	Error box area (deg ²)	Intensity (10^{-3} counts $\text{cm}^{-2} \text{s}^{-1}$)	Possible identification(s)
H0454+27	04 54.7 27°38'	0.65	2.9 ± 0.2	MWC*740 (B; $m_v = 12$)
H0500+24	05 00.8 24 58	1.80	2.0 ± 0.3	3C133
H0524+28	05 24.5 28 23	0.50	3.9 ± 0.4	1G*340(B) TMSS+30112 (B8)†
H0601+12	06 01.2 12 58	0.60	3.3 ± 0.6	1G*365(OB); TMSS+10105 (K5)‡
H0643+15	06 43.4 15 19	0.55	4.8 ± 0.7	—
H0649+17†	06 49 17 48	3.60	2.7 ± 0.6	NGC2304§; HD51354* (B; $m_v = 7$)

Parentheses indicate spectral classification and visual magnitude.

* Early-type star with emission lines (ref. 24).

† Marginal detection.

‡ $2 \mu\text{m}$ Sky Survey (ref. 25).

§ Compact stellar cluster.

association for four of the six sources is $\sim 10\%$. It would be worth a further investigation of these four stars to determine whether their temperatures are high enough to make them likely sources of X radiation.

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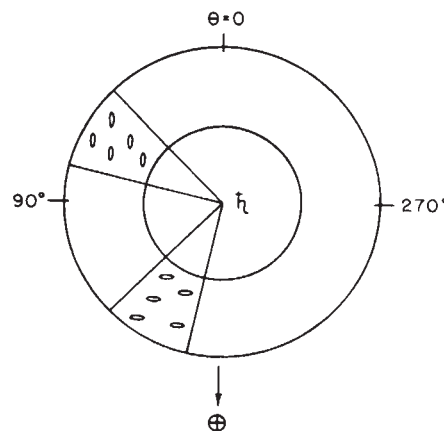


Fig. 1 Schematic view normal to the plane of Saturn's rings. Inhomogeneities in the distribution of particles, produced by mutual gravitation, are modelled as elongated 'blobs', which are orientated with their long axes inclined $\sim 70^\circ$ to the radius vector towards Saturn (blobs are shown for simplicity in two ring segments only).

11° – 12°), that the amplitude seems to decrease near opposition, and that the amplitude may decrease with decreasing wavelength (and hence decreasing particle albedo)^{11,12}.

We assume that the gravitational wakes or waves can be modelled as elongated distributions of particles which we shall refer to as 'blobs' (to distinguish these entities from individual ring particles). Each blob might result from a particle much larger than the average which has gathered wakes of smaller particles around itself; there would be many such blobs in a resolution element as viewed from Earth (Fig. 1). Radiative transfer in the rings will now involve both processes internal to the blobs (multiple scattering and mutual shadowing among the smaller ring particles, which may themselves be of centimetre or metre dimensions), and analogous processes among blobs.

To illustrate our main point, let us assume that any resolution element in the rings may be considered as a horizontally infinitely extended plane parallel layer in which the blobs constitute the scattering centres. For simplicity we furthermore assume that the blobs are optically thick when viewed from any angle. The reflected intensity from the rings may then be written⁷ as

$$I_\lambda(\theta; \mu, \alpha) = \frac{p_\lambda(\theta, \mu)}{4\pi a(\alpha)} F_\lambda(\mu, \alpha) \times [1 - \exp(-a(\alpha)\tau_\lambda(\theta)/\mu)] \quad (1)$$

where θ is azimuthal angle, τ is optical thickness, μ is the secant of the ring tilt angle with respect to the Earth (assumed for simplicity equal to the same angle with respect to the Sun), α is phase angle, p is the geometric albedo of a blob (actually the product of geometric albedo and phase function, but we ignore for the moment angular variations in the latter), λ is wavelength, F is a factor which accounts for the effect of multiple scattering between blobs (with an appropriate normalisation of the incident solar flux, $F = 1$ for negligible multiple scattering), and $a(\alpha)$ is a parameter to account for effects of mutual shadowing either between blobs or among the particles within a blob ($a(0) = 1$, $a \rightarrow 2$ as α increases). In principle, one can obtain azimuthal variations from equation (1) either through the effective optical depth $a\tau_\lambda$ as suggested by Franklin and Colombo¹⁰, or possibly through the multiple scattering (L. Esposito, personal communication). We propose instead to consider a new source for azimuthal variations, the geometric albedo p of the blobs.

The essential point is that the geometric albedo for a distribution of scattering particles considered as a single unit (p_{blob}) will vary with viewing angle. Take for simplicity the blobs to be

A model for the azimuthal brightness variations in Saturn's rings

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The precise nature of the rings of Saturn is poorly understood. There is controversy, for example, over whether the rings have relaxed into essentially a monolayer, or whether the observed sharp brightening near opposition confirms the classical model of mutual shadowing in a layer many particles thick^{1–3}. Likewise, the observations of azimuthal brightness variations in the rings (variations as a function of orbital phase of the ring particles)^{4–6} have been interpreted as implying the presence of large, synchronously rotating particles with either elongated shapes or systematic leading–trailing–edge albedo differences⁷ (although such synchronous rotation seems very unlikely⁸); or alternatively the presence of density waves analogous to those postulated for spiral galaxies⁹ or 'density wakes' resulting from mutual gravitation between a relatively large ring particle and nearby smaller particles. Here we present a model to explain azimuthal brightness variations in the rings. Assuming the existence of density irregularities as a result of self-gravitation, we outline the probable photometric effects. The results amplify the purely geometric discussion given by Franklin and Colombo¹⁰.

A successful model must explain not only the existence of azimuthal variations in ring brightness, but also the dependence of their amplitude on geometrical and photometric parameters. Thus, observations indicate that the effect is detectable in ring A but not in ring B (the latter being closer to the planet and having at least twice the optical thickness of ring A), that the amplitude in ring A increases as the slant optical thickness increases from ~ 1 to ~ 2.5 (as the tilt angle decreases from about 26° to

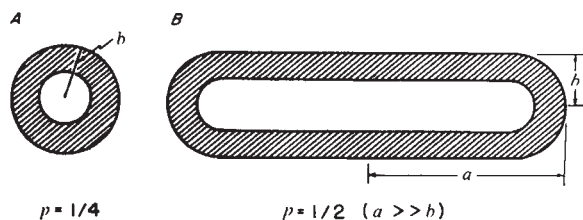


Fig. 2 Schematic representation of effect of limb darkening on geometric albedo p for an elongated distribution of particles considered as a single entity (a 'blob'). A, View normal to minor axis; B, view normal to major axis. Taking region within $b/2$ of limb to be perfectly absorbing (hatched area) and remaining area to reflect like a Lambert disk (local albedo of unity), geometric albedo in case B approaches a value twice that obtained in case A if dimension $a \gg b$.

figures of revolution about the major axis, and remember that the geometric albedo measures the flux reflected in the direction of the observer normalised by the projected area. Thus, p will decrease as limb darkening increases in importance. Limb darkening will be more pronounced when the blob is viewed along the major axis than when viewed normal to the major axis for two reasons (compare Fig. 2). First, in the approximation in which every surface element of the blob is replaced by a plane parallel layer so that its reflectivity is given by the appropriate H-function¹³, reflected flux will decrease for typical phase functions as the angles of incidence and reflection increase; this decrease occurs most rapidly when viewing the blob normal to its minor axis. Second, edge losses when viewing the blob 'end on' will be more significant, thus reducing the source function for multiple scattering below that which would apply if each surface element could indeed be approximated as plane parallel (as is normally done in radiative transfer calculations). Note that both these effects are the result of multiple scattering within the blob; were only single scattering present, the geometric albedo would be independent of orientation for an optically thick blob.

Let us compare these ideas qualitatively with the observations. Theoretical computations¹⁰ (also H. Karttunen, personal communication) show that the wakes which we have modelled as blobs will be orientated with their major axis inclined about 70° from the radius vector towards the centre of Saturn. Thus, the low albedo side will be orientated towards a terrestrial observer when θ (measured from superior geocentric conjunction) $\approx 70^\circ$, and p will be maximised when $\theta \approx 160^\circ$, in agreement with observation^{5,12} (compare Fig. 1). The dependence of the amplitude of the effect on tilt angle is also produced: if viewed normal to the ring plane all portions of the ring are equivalent and no effect would be observed; as the tilt decreases, however, the low albedo face of the blobs gradually becomes visible, and the effect grows in magnitude until deviations from a monolayer distribution of the blobs or the effect of an inter-blob background distribution of particles becomes significant. The dependence on phase angle and the possible dependence on wavelength also follow; in both cases a decrease in the importance of multiple scattering occurs, thus leading to a decreasing amplitude for the azimuthal variations according to the model described in the previous paragraph. In the case of the phase angle variation, we attribute the relative increase in single scattering near opposition to mutual shadowing among the particles within a blob, just as in the classical explanation for the opposition effect in Saturn's rings¹⁴. The wavelength dependence follows because the ring particle albedo decreases with decreasing wavelength throughout the visible spectrum^{15,16}, thus decreasing multiple scattering. In principle, the model would allow azimuthal variations in ring B as well as in ring A. That these are not seen implies that density wakes are not present, either because of proximity to Saturn and hence reduced relative importance of mutual gravitation among the ring particles, or perhaps because of different particle size distributions in the two rings.

The proposed blob model is clearly only qualitative and should be refined through both more detailed dynamical modelling of particle distributions and more rigorous calculations of the light scattering in such inhomogeneous layers. Such calculations might provide a measure of ring thickness through an analysis of the dependence of the azimuthal variations on ring inclination. More observational data obtained as the rings approach the edge-on position are now being analysed¹⁷.

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Energetic comets versus the Sun's companion

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The conjecture¹ that another star is near the Sun (at a distance R of the order of 10^3 AU) has provoked much discussion²⁻⁴. Subsequent work has tended to rule out all possibilities except for a neutron star or black hole making a high-speed, transient encounter with the Sun. I show here that due to the gravitational pull of such a companion, a large fraction of the comets coming into the Solar System from distances $\geq 40,000$ AU, that is $\gg R$, would arrive with positive energy ('hyperbolic') orbits. In reality, however, the original incoming orbits (with planetary perturbations compensated) are seldom hyperbolic.

Noticing that there are six pulsars with anomalously low spindown rates, and that these all lie in the general direction of the Galactic Centre, Harrison¹ pointed out that their low period-derivatives need not be an intrinsic property. Rather, a nearby star at distance R and of mass m_c could be causing the Solar System barycentre (or 'sun' for short) to accelerate in that direction with $g_c = Gm_c/R^2 \sim 10^{-6} \text{ cm s}^{-2}$; the steadily increasing Doppler blueshift would then be large enough to offset the normal decrease of pulsations and make the period derivative look small, or even negative. Henrichs and Staller³ later ruled out dwarf stars and planets (shining by reflected light) as being too luminous to have escaped detection. But Pineault⁴ showed that a neutron star or a black hole could indeed be permissible provided the (X-ray and visible) luminosity from accreting interstellar gas is cut down by suitable constraints, such as the companion passing the Sun and gas in a high-speed encounter, with, for example, $v_c \sim 10^2 \text{ km s}^{-1}$.

These authors dismissed a weakly-bound binary as being too liable to disruption. The size of unexplained residuals in

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Neptune's longitude (discussed in ref. 5) also limits a bound companion. When it circles at distance R , the tidal force it exerts on Neptune exceeds the known value unless $R \geq 15,000$ AU. Taking R as an average effective distance, the same conclusion should hold for any companion, bound or unbound, provided the interaction time (see, for example, equation (3)) is ≥ 100 yr, the time spanned by the Neptune observations. The elementary dynamical considerations of this paper demonstrate that a fast-moving companion cannot exist in a range of R roughly from 500 to 10^4 AU. The same approach might also work for a low-speed or bound companion, but simple analytical estimates are not easy to come by. Our argument holds independent of any assumed theory of cometary origins, be it Oort's⁶ giant 'comet cloud', an exploded planet⁷, or any other theory.

A comet (K) at distance r_C from the companion (C) and r_O from the Sun, changes its kinetic energy (per unit mass) because of the work performed on it by the two stars:

$$\Delta(v_K^2/2) = w_{OK} + w_{CK} = \Delta(Gm_O/r_O + Gm_C/r_C) - w_{KO} - w_{KC} \quad (1)$$

Here Δ denotes the amount of increase, and w_{ij} is the work done (per unit comet mass) by body i on body j . A distantly incoming comet makes its apparition at a distance of several AU and hence we can set its final $r_C = R$, the separation between the two stars; as the large initial distances $r_O \geq 4 \times 10^4$ AU, $r_C \geq 3 \times 10^5$ AU give negligible potential terms, the comet's final energy relative to the Sun reduces to

$$v_K^2/2 - Gm_O/r_O = Gm_C/R - w_{KC} - w_{KO} + v_{KO}^2/2 \quad (2)$$

where v_{KO} denotes the initial comet speed at large distances. The Sun's rest frame is accelerated by the attraction of the companion. Equations (1) and (2) hold in any inertial frame, but we use that one which coincides with the Sun's rest frame at the time of apparition.

A high-speed encounter with C incident, say, at $v_C = 10^2$ km s⁻¹ and having impact parameter $D \leq R$, lasts for a time of the order of

$$T = 2D/v_C = 10^2(D_3/v_{C2}) \text{ yr} \quad (3)$$

where D_3 is $D/(10^3$ AU) and v_{C2} is $v_C/(10^2$ km s⁻¹). A completed encounter imparts to the Sun (in the impulse approximation) a velocity

$$\Delta \tilde{v}_O = (2Gm_C/Dv_C) \hat{D} = 3 \times 10^{-2} \text{ km s}^{-1} (D_3/v_{C2}) \hat{D} \quad (4)$$

where $\hat{D}$ is the unit vector from the Sun to C's point of closest approach, and $g_C = 10^{-6}$ cm s⁻². If $m_C \geq m_O$, then C's change in velocity is even smaller and it thus deviates but little from uniform rectilinear motion.

The work to be evaluated is

$$w_{KO} + w_{KC} = \int \tilde{f}_{KO} \cdot \tilde{v}_O dt + \int \tilde{f}_{KC} \cdot \tilde{v}_C dt \quad (5)$$

where $\tilde{f}_{Ki}$ is the specific force exerted by the comet on star i . As $\tilde{v}_O$ is referred to the present, $t = 0$, rest frame of the Sun, and therefore goes to zero, we approximate it over the interval $-T \leq t \leq 0$ of the encounter by

$$\tilde{v}_O(t) = (2Gm_C/Dv_C) (t/T) \hat{D}$$

And we approximate the comet's orbit as an unperturbed zero-energy radial infall to the Sun because its distance $r_O(t) = 5.6 (-t/\text{yr})^{2/3}$ AU at the start of an encounter $T \sim 100$ yr ago was only about 120 AU $\ll R$. An integration by parts yields for the first integral in equation (5)

$$w_{KO} = -(Gm_C/D)(r_{Oi}/3D) \cos \theta \quad (6)$$

where $r_{Oi} = r_O(-T)$ and θ is the comet's angle of incidence relative to $\hat{D}$. The second integral in equation (5) is given by

$$w_{KC} = \alpha Gm_C/R \quad (7)$$

where $\alpha = v_{C||}/(v_{C||} - v_{K||})$ and $v_{C||}$, $v_{K||}$ are typical (signed) line-of-sight velocities from K to C at points of close approach between these two bodies. The coefficient α is ≤ 1 for comets approach-

ing the Sun from a direction roughly opposite that of the companion. For these comets, equation (2) becomes

$$v_K^2/2 - Gm_O/r_O \geq (Gm_C/D)(r_{Oi}/3D) \cos \theta + v_{KO}^2/2 \quad (8)$$

The incoming comet energy must therefore be positive for directions of incidence more or less opposite that of C, and within the hemisphere $\theta < 90^\circ$ centred about C's direction of closest approach. Thus we can be sure that at least something like a quarter of the distantly incoming comets must arrive with positive energy, but a larger fraction is possible, depending, for example, on the magnitude of the initial speed, v_{KO} .

The $\cos \theta$ term in equation (8) is not too large. However, the predicted abundance of hyperbolic orbits is unacceptable. Marsden⁸ found (out of 207 long-period comets) only one whose 'original' (incoming, prior to planetary action) energy had a positive value exceeding three times the formal error. In a thorough study, Z. Sekanina⁹ found out of 88 orbits five such hyperbolas. But at least two of these five are spurious, owing to the rocket thrust of outgassing (Whipple jet effect). Inclusion of the outgassing force usually does make the original orbit less hyperbolic¹⁰.

A fast-moving neutron star close enough ($R \leq 10^3$ AU) to produce $g_C = 10^{-6}$ cm s⁻² is thus excluded. But a supermassive black hole in a certain range of masses, for example, $m_C \sim 350 m_O$, $R \sim 1.5 \times 10^4$ AU, $v_C \sim 10^2$ km s⁻¹, cannot yet be definitely eliminated as a possible companion on the basis of its brightness or its perturbation of comet and planet orbits.

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A possible origin for the grooves of Phobos

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The surface of Phobos, the inner satellite of Mars, is marked by a series of linear depressions or grooves. These have been interpreted as surface manifestations of internal fractures^{1,2}. The origin of these fractures has been ascribed to tidal stresses induced by the decay of Phobos' orbit³, or to a nearly catastrophic cratering event². I suggest here that the age and distribution of the grooves may be explained by a hybrid origin due to varying tidal stresses induced after a large impact altered the satellite's rotation rate.

The orbital decay hypothesis was suggested by Soter and Harris³, who noted that Phobos is subjected to increasing tidal stresses as it approaches Mars. As the rate of orbital decay increases with time, this mechanism would require the grooves to be extremely young features. Later imaging at higher resolution revealed an appreciable number of impact craters superimposed on the grooves. Their implied age is $> 10^9$ yr (ref. 2), effectively ruling out this explanation.

Thomas *et al.*² noted that the grooves are most prominent near the largest crater, Stickney, and concluded that the fractures were produced by that impact. However, the relationship of the grooves with respect to the crater is not simple. They are neither concentric nor radial to the crater; rather, they seem to

define three sets of planes: one parallel to the equatorial plane, another perpendicular to the longest axis of the satellite, and a third intermediate between those two^{1,2}. While crater counts show that all of the grooves have roughly the same absolute age, some of them show cross-cutting which implies that they did not form simultaneously¹. If the material excavated from Stickney went into orbit about Mars, it should have been re-accreted by Phobos⁴; if uniformly distributed, it would blanket the surface to a depth of about 20 m. Many of the grooves exhibit relief of <10 m without apparent mantling by debris¹, suggesting that they were formed or renewed after the bulk of Stickney ejecta had settled.

Apparently then the existence and location of the grooves are related to the Stickney impact, but they formed shortly after that event, with orientations determined by the shape of Phobos. That shape is far from spherical, resembling a triaxial ellipsoid. The present rotation period of Phobos is equal to its orbital period. The longest axis of the satellite points towards Mars; this orientation minimises the internal stresses due to the combination of topography and tidal forces. These conditions almost certainly prevailed before the formation of Stickney, as the time scale for tidal despinning is <10⁶ yr (ref. 5). If the impact destroyed the condition of synchronous rotation, or simply induced a large libration amplitude, the changing orientation of Phobos in the gravitational field of Mars would subject its interior to periodically varying stresses. These could cause faulting along planes of maximum shear stress, producing the systems of grooves.

A quantitative estimate shows that this mechanism is plausible. In the stable synchronous state, the rotational energy is $Cn^2/2$, where C is the maximum moment of inertia and n is the mean motion. The criterion for destroying synchronicity, producing circulation rather than libration, is a change in rotational energy of magnitude $3(B-A)n^2/2$, where A and B are the moments of inertia about the other principal axes⁶. Modelling Phobos as an ellipsoid of mass 10^{19} g and semi-major axes of 13.5, 10.8, and 9.4 km, $C \approx 6 \times 10^{30}$ g cm², and $(B-A)/C \approx 0.25$. The change in rotational energy required to break synchronicity is, coincidentally, of the same order as the total rotational energy in the synchronous state. At present, these quantities are of the order 10^{23} erg. They vary as r^{-3} , where r is the orbital radius; as the orbit of Phobos is decaying, they may have been smaller in the past by one order of magnitude.

Thomas estimates the energy of the Stickney event at about 10^{26} erg. This figure should be regarded as having an uncertainty of at least an order of magnitude. In any case, only a small fraction of the impact energy would need to be partitioned into rotational energy of Phobos to remove it from synchronicity. For an impact energy of 10^{26} erg, at a velocity of 10 km s⁻¹ and an impact parameter of 10 km, the angular momentum imparted is $\sim 10^{26}$ g cm² s⁻¹. The present rotational angular momentum of Phobos is $\sim 10^{27}$ g cm² s⁻¹, but varied in the past as $r^{-3/2}$. Within the uncertainties of the estimate, the change in rotational angular momentum could have been comparable to total pre-impact value. In that case, the spin axis could have been appreciably displaced from the axis of maximum moment of inertia. The resulting nutation might account for those grooves which are not aligned with the principal axes. It is unlikely that the lesser impacts which Phobos experienced could have removed it from synchronous rotation; in this sense, the Stickney event was unique.

The excess rotational energy dissipated within Phobos during despinning after the impact was much less than the impact heating, and would have been negligible if uniformly distributed. However, it was presumably released along planes of faulting, and might have aided the escape of volatiles from the grooves¹. The time scale for despinning is $\sim 10^4$ – 10^6 yr (ref. 5), while re-accretion of ejecta orbiting Mars would take $\sim 10^2$ yr (ref. 4). Hence, it is reasonable that the grooves show no signs of burial by ejecta from Stickney.

Since Phobos shows no recent cracking in its synchronous state, the present stresses must be less than those produced

during non-synchronous rotation. For a tidally stressed elastic sphere⁷, the maximum tensile and shear stresses would be a few times 10^4 dyn cm⁻² near the surface, with the centre in compression at $\sim 10^6$ dyn cm⁻². These values are probably over-estimates, as when the long axis is towards Mars, Phobos is nearer to hydrostatic equilibrium than a spherical body would be (its present shape would be almost exactly in equilibrium at 1.25 times its present distance from Mars⁸). Even though Phobos is within the classical Roche limit for a liquid satellite (as its density is less than that of Mars), the stresses within it are quite small. However, in non-synchronous rotation, the topographic relief of ≈ 3 km would produce shear stresses of the order of a few times 10^5 dyn cm⁻² (ref. 9). The tidal stresses would be added to this topographic stress when the long axis of the satellite was pointed away from Mars.

The region opposite Stickney shows few grooves; therefore a shear strength $> 10^5$ dyn cm⁻² is implied for the undisturbed material. This value is consistent with a carbonaceous chondrite composition, but a stronger material cannot be ruled out. The region near the crater is presumably weaker due to fracturing by the impact, and may not constrain the properties of the pristine material. The low strength implied is for repeated loading; the yield strength under unidirectional stress may be greater. More realistic calculations of the stress field in a tidally distorted, ellipsoidal elastic body are needed to refine these strength estimates, and to determine whether the groove orientations are indeed consistent with this origin.

If the grooves are simply due to impact fractures, then such features might be common on asteroids, as predicted by Thomas *et al.*² If a strong tidal gravity field is also necessary, as suggested here, then only close satellites could possess regular systems of grooves. The absence of grooves on Deimos could then be due to its greater distance from Mars and smaller size, which resulted in lower stresses, or lack of an impact large enough to remove it from synchronous rotation. The best additional candidates for grooves would be Amalthea, the inner satellites of Saturn, and possibly the larger particles in planetary rings.

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The proton half life and the Dirac hypothesis

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Many notable physicists have been fascinated by the ubiquity of large dimensionless numbers formed from the physical parameters controlling the large scale structure of the Universe^{1–10}. For example, whereas most local dimensionless constants are within an order of magnitude or so of unity, the ratio of the electric to the gravitational force between a proton and an electron is $\sim 10^{40}$ whatever their separation; the number of protons in the Universe is $\sim 10^{80}$ and the ratio of the action of the Universe to the quantum of action, $\hbar$, is $\sim 10^{120}$ and so on. One stimulating approach to these large number coincidences was that introduced by Dirac⁵ in 1937 which is used here to predict precisely the proton half life. However, unlike previous applications, this prediction can be both tested and ruled out by experimental evidence.

Dirac argued that since the large numbers constantly appearing in physics and cosmology are all simple powers of $\sim 10^{40}$ it is reasonable to suppose that they are simply related. This was proposed in the 'large numbers hypothesis' (LNH): "Any two of the very large dimensionless numbers in nature are connected by a simple mathematical relationship in which the coefficients are of order of magnitude unity". To maintain this Dirac treated the gravitational coupling 'constant' as a time-variable parameter. This speculation has created many new areas of investigation in both experimental and theoretical physics, for example: (1) detailed experimental tests of planetary dynamics¹¹ to limit the possible time variation of the newtonian gravitational constant, G ; (2) the development of alternative theories of gravity like that of Brans and Dicke¹²; and (3) the introduction of an anthropomorphic mode of explanation which connects these large ratios to those features of the Universe which, superficially at least, render our existence possible⁶. Although these theoretical and experimental offshoots have been important in the understanding of gravitation physics, they all suffer from a common defect. They do not generate experimental predictions which would allow us to rule out the LNH. Some, like Eddington's 'fundamental theory'², provide only formal and predictionless relationships between otherwise unrelated quantities. Others, which incorporate time-varying 'constants' can be constrained by observations but never ruled out as the variations allowed may be made arbitrarily small by a suitable choice of the free parameters of the theory.

This letter presents a clearcut and virtually inflexible prediction of the LNH in its strongest form wherein large dimensionless numbers of equal magnitude are taken as precisely equal. This prediction allows the LNH to be ruled out by experiment. The basis of this prediction is the recent development of grand unified theories of the electro-weak and strong interactions¹³⁻¹⁶. Such a unification is possible at extremely high ($\sim 10^{15}$ GeV) energy when based on simple gauge group symmetries¹⁵ like SU(5), SO(10) or E_6 , and at much lower energies $\sim 10^5$ GeV when based on semi-simple¹³ [SU(4)]² gauge groups with chiral colour. The mass M_x at which unification takes place is given roughly by a relationship of the form¹⁶:

$$\alpha^{-1} - \alpha_s^{-1} \sim [I(S) - I(EW)] \frac{11}{6\pi} \ln \left(\frac{M_x}{\mu} \right) \quad (1)$$

where α_s and α are the low energy gauge constants of the strong (S) and electro-weak (EW) forces; $\mu \sim 80$ GeV this low energy, and I the Casimir operators for the residual, low energy strong and electro-weak symmetries. One of the consequences of these theories is the non-conservation of baryon number mediated by colour and charge-coupled lepto-quarks. This predicts proton decay ($p \rightarrow \mu^+ + \pi$ or γ in SU(5) or $p \rightarrow 3\nu + \pi$ in [SU(4)]²). In the SU(5) model a theoretical estimate¹⁵ of the proton lifetime, τ_p , lies in the range $10^{29} - 10^{37}$ yr.

$$\tau_p \sim \alpha_x^{-2} M_x^4 m_p^{-5} \frac{h}{c^2} \sim 10^{31} \left(\frac{M_x}{10^{15} \text{ GeV}} \right)^4 \text{ yr} \quad (2)$$

where α_x is the coupling between x bosons and fermions and m_p the proton mass. (Note that the value of M_x determined theoretically via equation (1) is exponentially sensitive to the other theoretical parameters and this 'instability' is further magnified when raised to the fourth power to determine τ_p via equation (2). This makes a precise prediction difficult.) A detailed discussion of the experimental status of these unified gauge theories may be found in ref. 17.

To connect these gauge theoretical ideas in particle physics to the LNH of Dirac, note that the dimensionless ratio of τ_p to the Planck time $t_{p1} = (Gh/c^5)^{1/2} \sim 10^{-43}$ s is of the order of the Eddington large number $\sim 10^{80}$. Assuming that the Universe is well-approximated by the closed Friedmann universe, we follow the LNH and equate τ_p/t_{p1} to the other invariant number of similar order, the number of protons in the closed Friedmann universe. (This is chosen rather than the number in the Hubble sphere today to avoid the conclusion that any 'constants' of

nature must vary in time.) This procedure predicts τ_p to high precision.

Using the standard results of general relativity (see, for example, ref. 18) and assuming zero cosmological constant (which is demanded by the large number hypothesis), it is possible to calculate the number of protons, $\mathcal{N}$, in the closed non-Euclidean geometry containing a pressureless gas of galaxies. The result depends only on two observable parameters: the present Hubble parameter, H_0 , and the present value of the universal density, Ω_0 , in units of the critical closure density, $\rho_c = 3H_0^2/8\pi G$

$$\mathcal{N} = \frac{3\pi}{4} f(\Omega_0) \frac{c^3}{Gm_p H_0} \quad (3)$$

where

$$f(\Omega_0) \equiv \Omega_0(\Omega_0 - 1)^{-3/2} \quad (4)$$

When equated to τ_p/t_{p1} this yields:

$$\tau_p = \left(\frac{hc}{Gm_p^2} \right)^{1/2} f(\Omega_0) \frac{3\pi}{4H_0} \quad (5)$$

Or, numerically:

$$\tau_p = 1.5 \times 10^{30} \text{ yr} \left(\frac{50 \text{ km s}^{-1} \text{ Mpc}^{-1}}{H_0} \right) \Omega_0(\Omega_0 - 1)^{-3/2} \quad (6)$$

Observations presently dispute the value of H_0 in the range $50 - 100 \text{ km s}^{-1} \text{ Mpc}^{-1}$ and the dependence on $\Omega_0 \sim 1$, ($\Omega_0 \geq 1$ in this 'closed' model) is very weak. Other small corrections might be anticipated as the Universe is not precisely homogeneous and isotropic.

The prediction (6) is interesting because present experimental evidence places limits of $\tau_p > 10^{29}$ yr for multi-body decays¹⁹ in which all three interior quarks decay and $\tau > 10^{30}$ yr for decays producing muons²⁰. Within 1 year proton decay on the time scale predicted by (6) should be detectable²¹.

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Rapid size measurement of sub-micron particles

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The evaluation of particle size in colloidal suspensions is of considerable academic and commercial interest. The greatest uncertainty lies in the measurement of particles of sub-micron dimensions. We report here a novel method for particle sizing in this range which is rapid, ideally suited to rigid particles and can be adapted to commercial situations. It is based on the induction and measurement of birefringence in a colloidal medium on its subjection to short pulses of high frequency ultrasonic power.

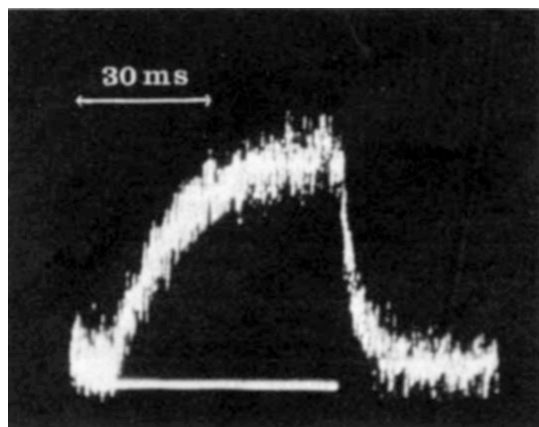


Fig. 1 Ultrasonically induced transient birefringence. Data for an 8% suspension of polytetrafluorethylene in water at $\sim 20^\circ\text{C}$. Maximum birefringence $= 1.6 \times 10^{-7}$. The horizontal line indicates the duration of the applied ultrasonic pulse, which was of 11.4 MHz frequency, 50 ms duration and approximately 1.2 W cm^{-2} power density. Time runs from left to right.

Most colloidal particles are either optically anisotropic or anisodiametric, or both. In a dilute suspension, the particles adopt a random array and the medium as a whole is isotropic. Application of an ultrasonic beam to the suspension subjects the suspended particles to a radiation pressure with the result that the particles orientate to a partial or full alignment with their major dimensions perpendicular to the direction of propagation of the ultrasonic beam. This orientation phenomenon is that of the Rayleigh disk¹. The resulting orientational order of the suspended particles renders the medium birefringent.

Although it was 1936 when Burger and Söllner² observed particle orientation for several colloidal suspensions in acoustic fields, acoustic birefringence, or the Lucas³ effect as it is sometimes called, has had little application in studies of particulate suspensions. Preliminary measurements have been reported of the amplitude of the effect for bentonite⁴ sols and tobacco mosaic virus⁴ solutions. A factor that has been little appreciated⁵ is that ultrasonic fields can be generated as short duration rectangular pulses so that any orientational birefringence becomes transient in nature. It could build up from zero to a steady constant value for a pulse of sufficient amplitude only to decay back to its pre-field state when the pulse stops. The decay rate is then a measure of the rotary diffusion and hence of the size of the particles in their fluid environment.

We have assembled a birefringence apparatus in which the light from a helium-neon laser passes through a pair of

polarisers set in the 'crossed' mode before impinging on the photomultiplier. Between these polarisers is the test sol contained in a custom designed cell. This holds some 40 ml of suspension. The ultrasonic beam is introduced into the sample via a piezo-electric transducer which is set back in one side of the cell so as to send the ultrasonic pulse through the sample and transverse to the light beam. The polarising devices are positioned to an azimuth of 45° with respect to the ultrasonic pressure gradient. After traversing the sample, the ultrasonic wave is absorbed in an acetic acid reservoir. When the ultrasonic pulse is applied, the suspended particles orientate, the medium becomes birefringent and elliptically polarised light leaves the sample cell. This cannot be extinguished by the analysing polariser and so light reaches the photomultiplier whose transient output is displayed on an oscilloscope and recorded. The recording may be photographic or via a transient recorder direct to a dedicated processor. With the optical arrangement described above, the transmitted light intensity is a function of the square of the birefringence (Δn) induced in the sample. Full details of the apparatus will be given elsewhere. Figure 1 demonstrates the reality of transient ultrasonic birefringence. Data are for a dilute aqueous suspension ($8 \times 10^{-3} \text{ g ml}^{-1}$) of polytetrafluorethylene (PTFE) particles, subjected to an ultrasonic pulse of $\sim 1.2 \text{ W cm}^{-2}$ at 11.4 MHz frequency for some 50 ms duration.

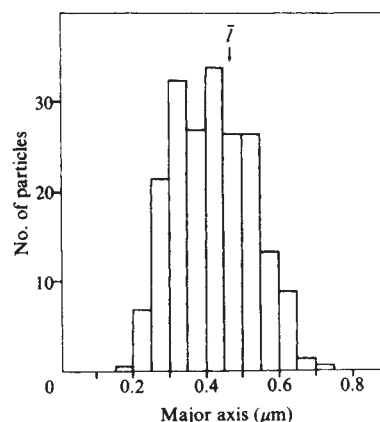


Fig. 3 Histogram of the major axes of PTFE particles. The arrow indicates that average value of the particle major dimension $\bar{l}$ obtained from the initial slope of Fig. 2.

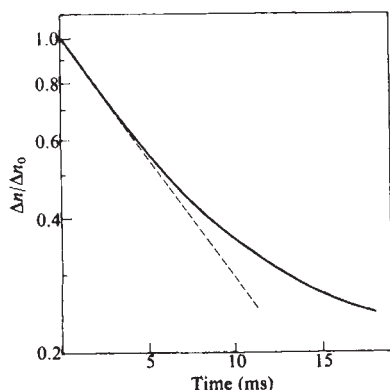
Particle size information is extracted as follows. The decay of the transient is digitised as a function of time. As the particle rotation follows a first order rate equation⁶, the birefringence (Δn) at any time (t) after the commencement of the decay obeys the following equation for a monodisperse sol,

$$\Delta n = \Delta n_0 \exp - 6Dt \quad (1)$$

Here Δn_0 is the birefringence amplitude when $t = 0$ and D is the rotary diffusion coefficient for the particles. This parameter can be obtained from the negative slope of a plot of $\ln(\Delta n/\Delta n_0)$ against t . Such a plot is shown in Fig. 2 for the birefringence transient of Fig. 1. The plot is seen to be curved, indicating the polydisperse nature of the PTFE sample, with each composite size species contributing to the recorded birefringence decay. A most convenient average value of D is obtained from the initial slope of Fig. 2 for which the data hold the least experimental error. For this sample $D = 25.5 \text{ s}^{-1}$. Auxiliary electron micrographs on this sample confirm its polydisperse nature as shown in Fig. 3. This was compiled from the measurement of some 200 particles. The particles were also seen to be well represented by prolate ellipsoids of some 1.5 axial ratio (r). For prolate ellipsoids⁷

$$D = \frac{3kT}{2\pi\eta l^3} \frac{r^4}{(r^4 - 1)} \left[\frac{2r^2 - 1}{r(r^2 - 1)^{1/2}} \ln \{r + (r^2 - 1)^{1/2}\} - 1 \right] \quad (2)$$

Fig. 2 Analysis of the transient birefringence decay. Data obtained from Fig. 1. The initial slope corresponds to $D = 25.5 \text{ s}^{-1}$ and a particle length of $0.47 \mu\text{m}$.



where l is the major axis of the particles, k is Boltzmann's constant, T the absolute temperature and η the solvent viscosity. The acute dependence of D on the major particle dimension l should be noted. It should be a good parameter for discriminating particles of different size and hence for the evaluation of particle dimensions. Using equation (2), together with the values of $D = 25.5 \text{ s}^{-1}$, $\eta = 9.2 \times 10^{-2} \text{ kg m}^{-1} \text{ s}^{-1}$ and $r = 1.5$ gives a particle length of $0.47 \text{ }\mu\text{m}$. A comparison with the histogram justifies this result.

We conclude therefore that by generating pulses of ultrasonic power and applying them to colloidal suspensions, transient birefringence can be induced in the suspensions. The rate of decay of the transient is very sensitive to the size of the suspended particles and affords a very fast and convenient measure of the same. Experiments are in progress to apply ultrasonic pulses of variable amplitude and duration as a means of evaluating a distribution of particle sizes. The ease of adaption of the method to industrial and 'on-line' size measurement is apparent.

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X- or γ -rays from supernovae in glacial ice

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In an analysis of NO_3^- in an Antarctic ice core we have found four spikes of high concentration, three of which occur at depths which correspond roughly to the dates of known galactic supernovae (SN). The production of the observed NO_3^- peaks by the hard X rays generated by a SN outburst (particularly Type I) does not seem inconceivable at least from the point of view of energy requirements and current SN models. We predict that the bright SN of 1006 will be 'observed' about 15 m beyond the end of the current core. If this is true, our identification of these spikes with SN will have important consequences for the theory of SN, atmospheric chemistry and transport, and even the dating of ice cores.

Two of us (E.J.Z. and B.C.P.) are involved in a project to determine NO_3^- and NH_4^+ concentrations in Antarctic glacial cores. The cores are taken from high altitude sites on the Antarctic Plateau where they are thought to be isolated from NO_3^- and NH_4^+ blown from the ocean. It has been pointed out¹ that a substantial fraction of the world's input of these molecules might be produced in the upper atmosphere via some mechanism which has at least modulated by the 11-yr solar cycle. Rather than concentrating on the more or less continual production, we discuss here some 'events' we have found recently.

Figure 1 shows the nitrate ion concentration plotted as a function of depth (from refs 2, 3). There are 864 contiguous data points. The experimental techniques will be described elsewhere. Three alternate chronologies are shown to give an idea of

the dating uncertainty. The bottom of the core corresponds to a date in the mid twelfth century. The NO_3^- concentration shows many noisy variations, some suggesting the solar cycle, particularly during the nineteenth century, and a pronounced minimum associated with the Maunder minimum⁴, a time of solar inactivity. Although current glaciological studies have been concerned primarily with long term variations, a new programme is in progress that will attempt to determine the significance of the short-term variability. The data plotted in Fig. 1 show four prominent spikes or events. A striking coincidence is that within the accuracy of the dating three of these events are at times of known supernovae: SN1604 (Kepler), SN1572 (Tycho), and SN 1181 (ref. 5).

The presence of an event in the first half of the fourteenth century is not surprising because even a bright SN at this time would possibly not have been recorded, particularly if it was in the south. There are several candidates SN remnants which might correspond to this event, including G278.5 – 0.5 (ref. 6) at declination -59° and MSH11 – 54 (ref. 7) at -54° .

Perhaps more disturbing is the absence of an event for the SN associated with the radio source Cas A. This SN is usually assumed to have occurred around 1700. (There is an increase in the local background at about that time, but no event.) The Cas A SN may have been significantly different from the others. It seems to have had a very massive progenitor and may have been Type II. Tycho and Kepler were Type I SN with low or moderate mass progenitors; the Type of SN1181 is not known for certain. Cas A was probably surrounded by a large amount of circumstellar gas at the time of the SN⁸. Although it is of comparable distance to the others and occurred when many observatories existed, it was not seen in visible light. It may well have been deficient in its output of visible light and, we conjecture, more energetic photons⁹.

For SN to have produced the observed events, there are several immediate requirements. We reject energetic particles as a source of the spikes as they travel along galactic magnetic field lines, and hence would arrive much later than the photon pulse. In fact, they are probably so spread out in time that they would not produce sharp events. Photons seem the only possibility.

Ruderman¹⁰ has considered the effects of very nearby SN on atmospheric chemistry and the possible destruction of the ozone layer. However, the historical SN are much too distant to produce these effects. As the spikes stand out above the background, the SN must stand out above the Sun in the flux of the NO_3^- producing photons. Typical SN energies are 10^{50} erg and distances are a few kpc, so a cursory examination of the solar output¹¹ reveals that we must be dealing with photons of energy ≥ 10 keV, where the total SN output intercepted on Earth exceeds the annual solar output. Photons with energies of 10–30 keV are absorbed at altitudes of 60–35 km; those with energies between 30 keV and a few MeV are absorbed at about 35 km (ref. 12). At this level the ionised fraction of the atmosphere is very small. Thus, most of the energy of these photons would ultimately be deposited in the atmosphere in the form of ionisations. The SN production of NO_3^- is probably initiated by the ionisation of N_2 . Again, because of the neutral atmosphere, molecule formation by the N_2^+ will dominate dissociative electronic recombination. The N_2^+ will start a series of ion–molecule reactions involving nitrogen oxides which, it is reasonable to assume, will ultimately produce NO_3^- . Simple reaction chains can lead to the production of one to two NO_3^- per ionisation.

To produce a typical concentration of $20 \text{ C}_{20} \mu\text{g l}^{-1}$ of NO_3^- , it must be produced and transported to Antarctica so that it falls near the pole at a rate of $2 \text{ C}_{20} \times 10^{15} \text{ molecules cm}^{-2} \text{ yr}^{-1}$. (For a typical background $\text{C}_{20} = 1$; for SN $\text{C}_{20} \sim \text{few}$.) Suppose one produces ϵ nitrate ions for each 16 eV (the ionisation energy of N_2) deposited in the atmosphere by high energy photons. Further, let f_t be a transport coefficient which gives the mean rate of arrival of NO_3^- near the pole compared with the terrestrial average production rate. There must be moderately efficient transport as we can observe Tycho, a northern SN. The

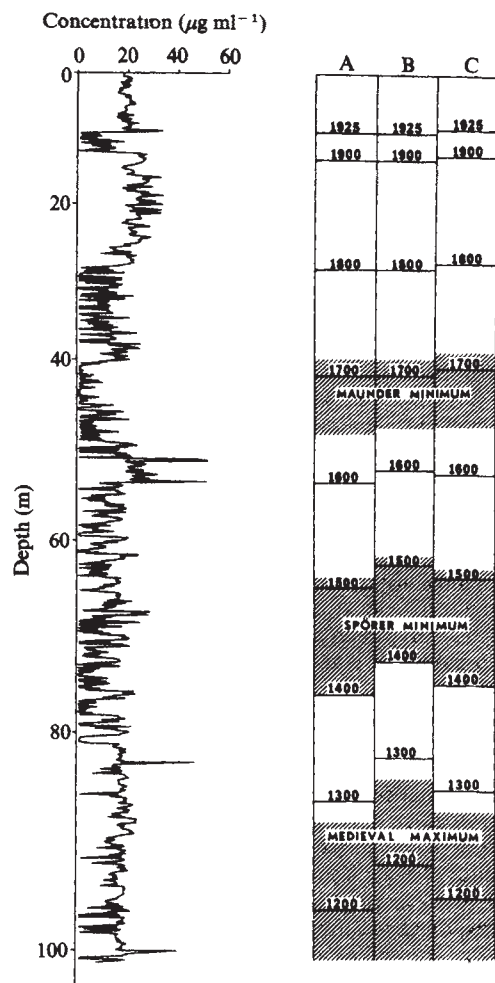


Fig. 1 Nitrate ion concentrations in the South Pole Station ice core.

atmospheric residence time must therefore be the order of weeks or months, but less than a year, as we observe events of short duration.) If NO_3^- falls preferentially in the polar vortex, then f_i could be >1 , but conservatively, we adopt $f_i \sim 1$. In terms of these parameters the energy flux required is:

$$F = \frac{1 \times 10^5 C_{20}}{f_i \epsilon} \text{ erg yr}^{-1} \text{ cm}^{-2}$$

For a SN of total energy output $E_{50} \times 10^{50}$ erg at a distance of d_{kpc} kiloparsecs from the Earth, $F_{\text{SN}} \sim 8 \times 10^5 \text{ erg cm}^{-2} E_{50}/d_{\text{kpc}}^2$. Most of the energy will be deposited in a few months. As the observed SN are at a few kpc, we see that our identification is reasonable only if $E_{50} \geq 1$. Further, as it is unlikely that $E_{50} \gg 1$, we also require that $\epsilon \sim 1$ while we have argued is plausible. (Note that we do not observe associated events in the NH_4^+ and would not expect to. The N in NH_4^+ is reduced while that in NO_3^- is oxidised, so it is likely that they have a different origin.)

Is $E_{50} \sim 1$ for photons ~ 10 keV reasonable? Klein and Chevalier¹³ predict X-ray bursts from Type II SN which are many orders of magnitude too small. The Type I SN we may be dealing with may be objects which have smaller envelopes and thus perhaps a harder and larger X-ray burst, but it is doubtful that the emerging shock such as that of Klein-Chevalier can give $E_{50} \sim 1$. Arnett (ref. 14 and refs therein) has argued that the long decay of the light curve of Type I SN could be the result of the decay of ^{56}Ni . If this is true, more energy comes out in the form of γ -ray lines than in visible light. Arnett finds E_{50} of a few tenths, but feels that $E_{50} \sim 1$ is not unreasonable (personal communication). It would be premature to identify the NO_3^- spikes with γ -ray lines from Type I SN. We only wish to point out

that the energetics of production are not out of line with some current SN models. On the basis of our model there is no distinction between any photons of energy from ~ 30 keV to a few MeV. If altitude is not crucial for production, the lower limit would extend to ~ 10 keV.

Note that the normal background production of NO_3^- may be by an entirely different mechanism. There could be some solar hard X-ray production and there certainly is ample energy in the softer solar X rays to make the NO_3^- at higher altitudes: either would show solar cycle and Maunder minimum like behaviour. If the SN identification for the spikes is verified, the fact that solar production does not overwhelm the SN spikes will show that production by photons of say 1 keV is much less efficient than that of more energetic photons either because of photon energy or altitude of absorption. The identification of the spikes with SN does not rule out auroral production for the background. Note that X- or γ -ray production would not be affected by the photodisintegration problems which may affect the auroral model^{15,16}.

Even though our identification of the NO_3^- events with SN is highly speculative, we can make some concrete verifiable predictions which will validate or discredit the identification. There are two recorded SN in the century and a half just preceding the present core. The first of these is the famous SN1054 or Crab; the second is SN1006. Chevalier¹⁷ has argued that the Crab is Type II, although it is still doubtful. If our failure to observe Cas A indicates that SN II do not produce NO_3^- at the Earth, then we may not see the Crab. Alternately Weiler and Panagia¹⁸ argue that the Crab remnant is very similar to that of SN1181 and that both are SN II so we may or may not expect a spike from the Crab. On the other hand, the case of SN1006 is clear. It was the brightest SN ever recorded¹⁹. Its high galactic latitude suggests that it was Type I. At a depth of 115 m near the South Pole or closer to the surface in an area of slower ice accumulation it should have left the largest spike of all in the NO_3^- concentration. If a NO_3^- spike is found at a depth roughly corresponding to AD 1006 a correspondence between NO_3^- spikes and galactic SN will be established.

A new firn core has been obtained from the South Pole Station and is being analysed at Virginia Polytechnic Institute for NO_3^- and NH_4^+ as well as certain other selected elements and ions. The new core will completely replicate the data on which this paper is based and possibly extend them to an earlier time. Smaller increments of ice can be analysed giving better time resolution.

This technique will open the possibility of many astronomical and atmospheric investigations. If NO_3^- in recent ice can be identified with an observed hard photon event such as a giant γ -ray burst, it will provide an absolute calibration and the technique becomes even more powerful. Fairly accurate estimates for the integrated hard photon luminosity of SN will be possible; this provides a strong test for theories of SN outburst. Even without an absolute calibration, the now very poorly known rate of galactic SN can be determined over a much larger period of time (at least for Type I). As the NO_3^- is produced by hard photons, the new rate will not be affected by the uncertainties due to interstellar extinction. If the difference between SN I and SN II is real, historical SN can be typed. Finally the confirmation of SN production of the NO_3^- spikes will assure that even extragalactic SN can be detected by present X- and γ -ray satellites. SN production of NO_3^- events will provide important information on atmospheric chemistry and circulation. Perhaps, most important is the demonstration that some NO_3^- production must be at an altitude of 35 km, probably resulting from ionisations occurring in the predominately neutral gas.

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Ross Ice Shelf temperatures support a history of ice-shelf thickening

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The ice sheet in West Antarctica is grounded on a sub-sea level basin in the Antarctic continental shelf. At the seaward margins, where ice thicknesses are reduced sufficiently to attain neutral buoyancy, floating ice shelves form. The two largest are the Ross and Ronne ice shelves (Fig. 1). Because the depth of the continental shelf increases towards the centre of the West Antarctic ice sheet, ice discharge could accelerate irreversibly until the grounded portion of the ice sheet had completely disappeared^{1–3}. The resistance to ice discharge provided by the ice shelves is the major factor preventing such a collapse^{4,5}. Geological evidence indicates that the extent of grounded ice in the West Antarctic has fluctuated widely during the Pleistocene^{6,7}, and complete removal of the ice sheet may have occurred during the Sangamon interglacial following a climatic warming which was probably associated with a retreat of the ice-shelf seaward margins⁸. One of the most active ice streams draining West Antarctica flows into the south-east corner of the Ross Ice Shelf. Surface observations in this area indicate that, either the ice shelf is thickening⁹, or that there is appreciable melting from beneath ice shelf that is >500 km from the open sea¹⁰. Such melting would strongly influence temperatures within the ice shelf, and here we use a temperature–depth profile from the ice shelf to estimate the basal regime. We conclude that, for much of the south-east corner, basal freezing/melting rates have been near zero for the past few hundred years. This implies that, within this region, the ice shelf is thickening at $\sim 0.3 \text{ m yr}^{-1}$.

Whether ice shelf at a fixed geographical location will thicken or thin with time is determined by the relative effects of snow accumulation rate $\dot{A}$, basal-freezing rate $\dot{B}$, creep thinning due

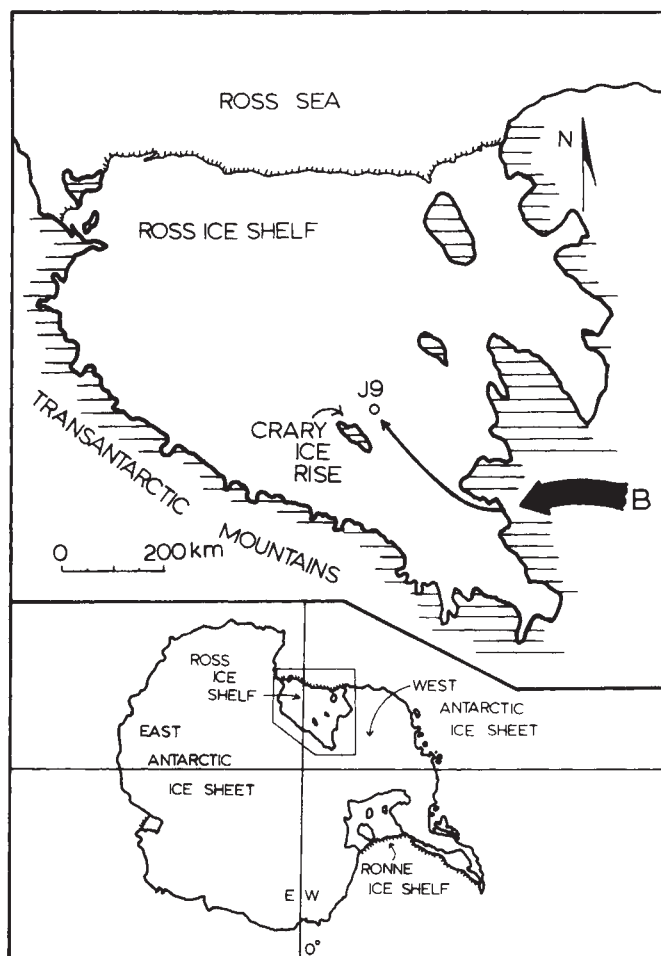


Fig. 1 The Ross Ice Shelf, showing the flowline from ice stream B to the Ross Ice Shelf Project drill hole at J9.

to the influence of ice-shelf stresses, and the advection of thicker ice from upstream. The ice-shelf thickening rate $\dot{H}$ is

$$\dot{H} = \dot{A} + \dot{B} + H\dot{\epsilon} - V \partial H / \partial x \quad (1)$$

where H is ice thickness, $\dot{\epsilon}$ is the vertical strain rate, V is the horizontal velocity of the ice shelf and $\partial H / \partial x$ is the thickness slope in the direction of movement. Measured values of $\dot{A}$, V , H , and $\dot{\epsilon}$ show that the current average value of $(\dot{H} - \dot{B})$ is $34 \pm 15 \text{ cm yr}^{-1}$ across the southeastern section of the ice shelf⁹, upstream from Cray Ice Rise (Fig. 1). For the ice shelf to be in steady state ($\dot{H} = 0$) would require a melting rate of $\sim 34 \text{ cm yr}^{-1}$ from beneath ice shelf that is >500 km from the open sea. Alternatively, a near-zero basal mass balance would indicate rapid thickening of a large area of ice shelf downstream from one of the most active West Antarctic ice streams (B in Fig. 1). This would imply advance of the West Antarctic ice sheet into the Ross Ice Shelf by $\sim 1 \text{ km yr}^{-1}$.

Oceanographic measurements from beneath the ice shelf via the Ross Ice Shelf Project drill hole (J9 in Fig. 1) suggest that basal melting occurs^{11,12}, but this result is more appropriate to average conditions between the seaward ice front and the drill hole. More recently, a core obtained from the ice shelf at J9 was found to include a bottom section of sea ice, 6 m long¹³. Details of the crystal structure of the sea ice suggest that basal freezing occurs at J9 and for at least some tens of km upstream from J9 (I. Zotikov, personal communication). Unfortunately, the area upstream from Cray Ice Rise, where the ice shelf may be thickening, is >100 km from J9. Moreover, we cannot assume that conditions become more favourable for freezing with increasing distance from the open sea, because there may be alternating bands of basal freezing and melting associated with

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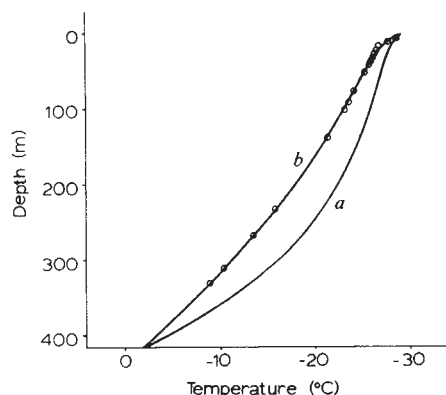


Fig. 2 Temperatures measured in the drill hole at J9, and two calculated temperature–depth profiles: (a), for a steady-state ice shelf with basal melting at about 30 cm yr^{-1} ; (b), for an ice shelf with basal freezing of 8 mm yr^{-1} , and thickening of $\sim 30 \text{ cm yr}^{-1}$ (○, measured). This value of the freezing rate is sufficient to give the 6 m of sea ice that was found at the bottom of the J9 ice core¹⁰.

the passage of ocean currents beneath large-scale undulations in the bottom of the ice shelf¹⁰. According to this model the J9 region could be in a basal-freezing zone, whereas further upstream there may be melting if southward flowing sea water is forced downwards to pass beneath the thick lobe of ice stream B. Consequently, we have attempted to interpret the temperature–depth profile measured in the ice shelf¹⁴ at J9 in terms of the basal conditions encountered by the ice as it moved through the southeastern section of the ice shelf towards its present position.

Heat flow in an ice shelf is generally directed vertically from the warm seawater at the base towards the cold polar air at the surface. Forced convection produced by vertical ice movement due to snow accumulation, vertical strain, and basal melting or freezing exists in addition to conduction. Using a finite-element computer model of time-dependent vertical heat flow¹⁶, we analysed the influence of convective heat-flux variations produced by basal melting and freezing on the temperature–depth distribution. We found that the basal melting or freezing previously encountered by the ice column had a strong influence on the curvature of its temperature–depth profile. Moreover, the time required for temperatures to reach equilibrium after a change of $\dot{B}$ was $\sim 500 \text{ yr}$. Thus, the present J9 temperature–depth profile should embody the average effect of basal melting or freezing previously encountered by the J9 ice column along most of its 800 yr since it began floating. The temperature–depth profile can be regarded as the ice-shelf memory, with most recent events recorded in temperatures nearest the upper and lower surfaces.

The finite-element model was used to simulate the temperature–depth profiles produced by prescribed conditions as the J9 ice column moved from ice stream B to its present position. Input parameters were values of $\dot{H}$, V , $\dot{A}$, $\dot{\epsilon}$, $\dot{B}$, and the surface and basal temperatures along the flowline upstream from J9. The initial temperature–depth profile for the point where the J9 ice column entered the ice shelf from ice stream B was calculated assuming steady state and with the same surface temperature, density–depth relationship, and snow accumulation rate as at J9, a basal temperature equalling the melting temperature of ice in fresh water at the appropriate pressure, and a basal melting rate of 5 cm yr^{-1} accounting for the basal shear and sliding of ice stream B. The effects of shear heating at the sides of ice stream B and of heat introduced into the ice shelf by bottom crevasses were not included. Output consisted of the calculated J9 temperature–depth profile. In the first test we used model input representing the present ice-shelf conditions with sufficient basal melting to balance equation (1) for zero thickening. This represents a ‘steady-state’ ice shelf in which the ice

thicknesses across the southeastern section were invariant with time. In these circumstances, the calculated J9 temperature–depth profile was much cooler than the measured profile (Fig. 2). The disagreement between the two profiles was due to the high basal melting rate necessary to preserve steady state. By relaxing this constraint and allowing the ice shelf to have thickened or thinned, we were able to fit the observed temperature–depth profile. Values of $\dot{A}$, the surface and basal temperatures, and the velocity of ice stream B where it enters the ice shelf were assumed to be the same as they are today; values of $\dot{H}$, $\dot{B}$, and $\dot{\epsilon}$, were allowed to vary within the constraints of equation (1). To accommodate migration of the grounding line between ice shelf and ice stream, the point where the J9 ice column first became afloat was moved 100 km downstream of the present grounding line for a thinning ice shelf and 100 km upstream for a thickening ice shelf. In all cases, the best agreement between simulated and observed temperature–depth profiles was obtained when the average value of $\dot{B}$ upstream of J9 was near zero. For a steady-state ice shelf, or for one that is thinning with time the values of the vertical strain rate $\dot{\epsilon}$ were found to be much higher than those currently observed. To obtain strain rates that approximated those of today the ice shelf was assumed to thicken at an average rate of 30 cm yr^{-1} during the time taken for the ice column to move from the grounding line to J9 (800 yr). The calculated temperature profile for this situation is shown in Fig. 2. Although there seems to be historical agreement between observed temperatures and strain rates, and the ice-shelf thickening in this area, the calculated thickening rate is unlikely to have been constant throughout the past 800 yr. Progressive thickening of the ice shelf has probably been associated with a progressive decrease in strain rates, so that our estimate of the thickening rate provides an upper limit.

Currently, there are six major ice streams flowing into the Ross Ice Shelf. Flowlines derived from present-day ice velocities in the Ross Ice Shelf indicate ice stream B as the origin of the J9 ice. However, if there have been large changes in ice velocities, the J9 ice may have come from an ice stream 150 km north of B. This would not affect our conclusion of near-zero basal mass flux and present-day ice-shelf thickening, but it would influence our interpretation of past ice-shelf behaviour. At the grounding line between ice stream B and the ice shelf, strain heating associated with both tidal bending and shear of the ice past its sides may have resulted in a warmer initial ice column than we assumed. However, unless the ice was at least 10°C warmer than we assumed, the effect on the temperature profile at J9 is $< 1^\circ\text{C}$. Also, although we have neglected non-vertical heat flow, radio-echo sounding has revealed bottom crevasses extending 100 m up into the ice shelf 200 m on either side of the drill site (ref. 17 and K. C. Jezek, personal communication). If these crevasses formed 800 yr ago when the J9 ice was near the outlet of ice stream B, then warming at the drill site due to the presence of seawater within the crevasses would be about 40% complete and would be $\sim 1^\circ\text{C}$. However, if these crevasses did not form until more recently as a response to flow past Crary Ice Rise, their effect on the J9 temperatures would be considerably less. At any event a perturbation of the temperature–depth curve by 1°C would not alter our general conclusion that the ice shelf has been growing thicker; it would simply shift the probable basal-flux regime to a low melting rate.

The near-zero basal mass flux predominance over most of the flowline upstream of the J9 drill site during the past few hundred years implies that the southeastern section of the Ross Ice Shelf has been thickening and continues to do so today. It is reasonable to suggest that thickening has occurred near the junction between the ice shelf and the grounded ice as well and that, locally, the junction has been advancing into the Ross Embayment. If this continues, large areas of the southeastern section of the Ross Ice Shelf will be replaced by grounded ice within the next 100 yr.

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Recognition of common Precambrian polar wandering reveals a conflict with plate tectonics

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The controversy concerning the applicability of plate tectonics to Earth processes during Precambrian time has centred on discussions of geological aspects of continental evolution, particularly the significance of mobile belts which seem to separate large tracts of cratonic nuclei^{1–4} and on analyses of palaeomagnetic data^{5–8} which could provide quantitative evidence defining the spatial relationships of the cratonic nuclei comprising continental mosaics. Briden⁹ has described the salient features of the plate tectonic model in relation to geological processes for Earth history in pre-Mesozoic time. The results of the published palaeomagnetic analyses for North America^{5,8,10}, Africa^{6,8} and Australia^{7,8} strongly suggest that the cratons which comprise the individual continents retained their relationships for much of Precambrian time and that the younger, intervening mobile belts and major sutures did not result from convergence of previously widely separated microcontinents; their origin is ensialic. The implication is that tectonic styles have changed through geological time⁸.

A landmass may be regarded as having existed as a single continent for a particular interval of time if the apparent polar wander paths (APWP) for the individual blocks or cratons coincide⁶. Collectively, they may be regarded as defining a master curve that describes the motion of the pole with respect to the continent. Of course, we rarely have a sufficiently complete data set to realise this ideal and we often need to resort to results from one block or craton to compensate for gaps in the record from other blocks. However, the overall continuity of the path is maintained and the known chronology of points on the APWP is not violated. On this basis APWP which have been constructed for North America, Africa and Australia have led to the conclusion that the three landmasses maintained their integrity for substantial amounts of Precambrian time. We have also included results from Greenland^{11,12} for the period from ~1,700 to 1,600 Myr, although these data only come from one craton and its surrounding mobile belts.

For the present analysis we take the comparisons a stage further. Accepting that continental integrity has been maintained, what can be deduced about the relative positions of North America, Greenland, Africa and Australia during the Precambrian? As we are primarily interested in making inter-continental comparisons, we are constrained to comparing APWP for the interval of time where defined sections of the

paths from more than one continent are available. In the time interval 2,300–1,600 Myr, which also covers the periods for which the palaeomagnetic data have been considered to indicate the existence of single continents, the APWP for North America has been constructed^{8,10,11,13} and can be shown to be continuous without significant time breaks. Similarly, paths have been constructed for Africa^{6,8} for the period 2,300–~1,900 Myr and for Australia^{7,8} from ~1,800–1,600 Myr, again supporting single continent models.

Figure 1 shows the North American and African data for the period 2,300–1,900 Myr and Fig. 2 compares the data for North America, Greenland and Australia for the period 1,800–~1,600 Myr. The quality of the data has been discussed elsewhere^{7,10,11,14} and reliability criteria have been implemented to designate pole status. The following discussion is based on a comparison of those data independently produced by a number of research groups using their own reliability framework.

The poles for the interval 2,300–1,900 Myr from North America and Africa respectively (Fig. 1) may be considered to define the same general track. The track for North America seems to be displaced a few degrees south of the track for Africa. This is a second order observation and may require further assessment.

The pole tracks for North America and Australia respectively coincide for the interval 1,800–1,600 Myr (Fig. 2). The tracks for Australia⁷ and Greenland¹¹ have been drawn with a northward trend from ~1,700 to 1,600 Myr.

The North American track has previously been drawn to extend into the eastern Pacific Ocean at ~1,500 Myr^{13,15}. However, this interpretation was based largely on an age of 1,500 Myr for the Western Channel Diabase. Subsequent radiometric evidence¹⁶ no longer justifies extending the North American track westwards. Recent re-evaluations of the North American data^{17,18} have led to similar conclusions.

The observation that the polar tracks coincide is made without applying any continental reconstructions. The geocentric angle subtended from a point fixed within one continent to a point fixed within another continent (North America and Africa for the interval 2,300–1,900 Myr; North America, Greenland and

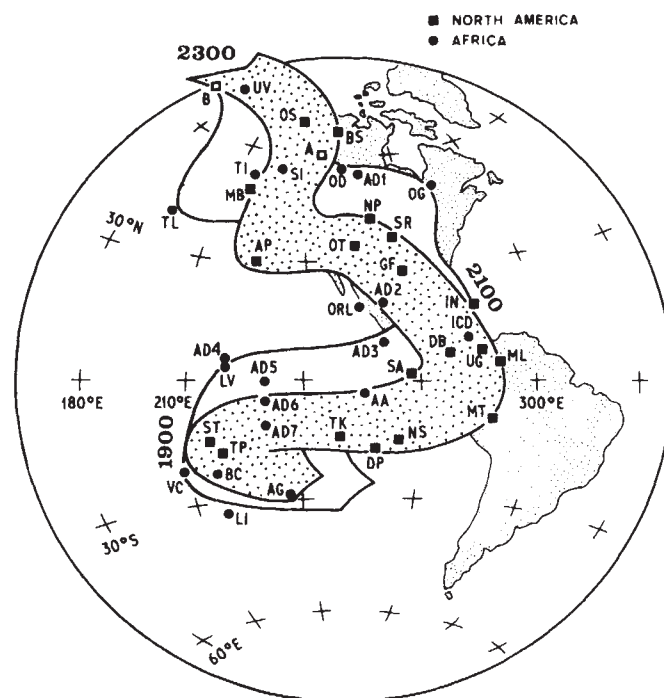


Fig. 1 APWP for Africa (unstippled) and North America (stippled) for the interval 2,300–1,900 Myr. Pole positions are identified for North America^{13,15} and Africa (ref. 8 following Piper²⁴) using mnemonics to represent rock formations. Note poles A and B (open symbols) plot on the obscured hemisphere.

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Cenozoic sedimentation in the central North Pacific

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The central North Pacific, one of the largest sedimentary provinces in the world ocean, is covered by red clays and abundant manganese nodules^{1,2}. Few studies have focused on the sedimentation history in this area, presumably because of the difficulty of dating these sediments; biostratigraphically useful fossils are rare or absent, and palaeomagnetic stratigraphy is limited in this area to the past 2–3 Myr (refs 3–5 and R. A. Prince *et al.*, in preparation). We present here a history of Cenozoic sedimentation for the central North Pacific based on lithologic, stratigraphic and sedimentological data from a single 25-m long giant piston core (B.H.C. and C.D.H. in preparation). A sedimentation model incorporating the present-day sedimentation patterns in the central North Pacific and the north-northwest movement of the Pacific plate during the Cenozoic seems to explain the observed sedimentological variations seen in this unique core.

Geological formations in the mid-plate, mid-gyre (MPG) regions of the ocean have been suggested as possible sites for the disposal of high-level nuclear waste⁶. In 1974 a study was initiated to assess the sub-seabed in the mid-plate, mid-gyre region of the North Pacific (MPG-1) as a potential repository for high-level nuclear waste (ref. 7 and our work in preparation). A more complete presentation of the data will be published elsewhere.

The MPG-1 area is centred at about 31°30' N, 158° W between the Murray and Mendocino fracture zones 1,100 km north of Hawaii, and lies on pre-Cenozoic crust of the magnetic quiet zone⁸ with no teleseismic activity reported⁹. The region is marked by low abyssal hills or swales with most of the region having water depths of 5,800–6,000 m (ref. 10). Red clay with grain sizes of about 1 μ m is the dominant sediment type¹, with calcium carbonate being less than 10% of sediment weight in the North Pacific¹¹. The circulation of Pacific bottom water ($\theta = 1.0$ – 1.1 °C) is sluggish^{12,13}, with current speeds rarely exceeding 10 cm s⁻¹, and mean speeds of about 1 cm s⁻¹ (refs 14 and 15). Tidal flow dominates the current records.

One giant piston core, GPC-3 (30°19.9' N, 157°49.4' W; 5705 m) was taken on Long Lines cruise 44 (Fig. 1). The core consists of a brown lutite ('red clay') with several altered ash layers. The colour of the sediment from 0 to 632 cm is a light yellowish brown and from 632 to 2,431 cm, a dark reddish brown. Calcium carbonate percentages range from 0 to 4%, indicating that the core site has been below the calcium carbonate compensation depth throughout the Cenozoic. Seven ash layers are present in the core; the ash layer at 1,077 cm has a 0.1-cm thick manganese crust on both sides, and the ash layer at 1,135 cm has a partial coating (<0.1 cm) of manganese.

The palaeomagnetic inclination data (Prince *et al.*, in preparation) are correlated with the palaeomagnetic stratigraphy of Cox¹⁶ from the top of the core to the Matuyama–Gauss bound-

dary (2.43 Myr ago). Below this level, the correlation breaks down, the DRM decreases in intensity and there is a change in the sediment colour. Based on the palaeomagnetic data, the sedimentation rates are 2.5 mm per 1,000 yr for the last 2 Myr and 1.1 mm per 1,000 yr before that to 2.4 Myr ago (Prince *et al.*, in preparation). This increase in Pleistocene sedimentation rates has been suggested to be due to the increase in glacial erosion, providing more material for transport than in previous times^{17,18}. Ichthyolith stratigraphy used to estimate ages of pre-Pliocene sediment indicates sedimentation rates of 0.2–0.3 mm per 1,000 yr from 65 to 5 Myr ago (P. S. Doyle and W. R. Riedel, in preparation). The ichthyolith stratigraphy is based on the distribution of fish skeletal debris in DSDP cores^{19–21}. The distribution of the ichthyoliths has been related to established biostratigraphic sequences within these cores to establish a chronostratigraphic framework (Doyle and Riedel, in preparation).

Size analysis of the sediment with a Coulter counter shows that mean grain sizes of the >0.63- μ m to <13- μ m fraction vary from 1 to 4 μ m. A 30- μ m aperture tube yielding a range from 0.63 μ m to 12.7 μ m was used because the >13- μ m fraction generally comprised <2% of the samples. Down-core trends in the finest grain sizes are not meaningful because of problems with flocculation during the analysis of the sediments. Sediment was wet sieved at 38 μ m and dry sieved at 63 μ m. The >38- μ m weight percentage ranges from <1 to 2% of the total dry sediment weight, and the >63- μ m fraction is <1% of the sediment weight throughout the core.

X-ray analysis of the <20- μ m fraction reveals that the sediment between approximately 24 and 10 m (65–22 Myr ago) consists of smectite, phillipsite, feldspars and clinoptilolite with a minor amount of quartz. From 10 m to the top of the core (22 Myr ago to the present), quartz, smectite, chlorite, cristobalite, kaolinite, illite, feldspars, mica and anatase are found.

The >38- μ m size fraction was examined with a light microscope to determine the sediment composition. Sediment between 24 and 10 m is composed primarily of fish debris, with minor amounts of detrital quartz, manganese micronodules and phillipsite. Mica is found in trace amounts between 24 and 12 m. Sediment between 10 m and the top of the core consists largely of manganese micronodules with common fish debris. Unidentified opaques are found throughout the core in trace amounts. The transition between the two sediment types at 20–30 Myr ago (9–12 m) is marked by large numbers of fragments of an agglutinated benthonic foraminifer, *Bathysiphon* sp., zeolite crystals and aggregates of zeolite crystals.

The sediment distribution patterns in the central North Pacific^{1,2,22,23} have been well documented. Manganese nodules are found north of 10° N, and are generally associated with non-calcareous clays. The dominant clay mineral in the central North Pacific between 20 and 40° N is illite which comprises >70% of the <2- μ m size fraction. Smectite is associated with abundant zeolites, and makes up 20–30% of the <2- μ m fraction between 20 and 40° N, increasing southward to values >70%. Chlorite contributes 10–20% of the <2- μ m fraction between 20 and 40° N, and decreases to <10% in most areas south of 20° N, although values between 10 and 20% can be found. Kaolinite contributes 5–20% of the <2- μ m fraction between 20 and 40° N, and has a more irregular distribution than the other clay minerals. Quartz shows a maximum concentration of 20% of the sediment at 30° N and decreases to the north and south.

The sediments can be divided into two assemblages (Fig. 2). (1) North of approximately 20° N the sediment contains a large detrital component as well as some authigenic components. This assemblage, referred to as the detrital assemblage, includes illite, chlorite, kaolinite, quartz, manganese nodules and smectite. It results largely from eolian transport of continental detritus by the jet stream or surface winds (westerlies) and reflects the amount of exposed arid regions of the Northern Hemisphere^{22–24}. (2) Between approximately 20° N and 10° N the sediment is composed primarily of authigenic components such as smectite and manganese nodules, and is referred to as the

authigenic assemblage. This mineralogical boundary at approximately 20° N is defined by the regional sedimentation patterns of two important minerals, illite and smectite. Illite has values between 50 and 60% north of 20° N latitude, and rapidly decreasing values south of 20° N. Smectite has low values of 20–30% north of 20° N and increasing values south of 20° N.

A sedimentation model based on the north-northwest migration of the Pacific plate during the Cenozoic through regions with different sedimentation characteristics (Fig. 2) is proposed to account for the sedimentological variation in GPC-3. The assumption is made that the sedimentation processes during the Cenozoic were similar to those found at present and that the zonal distribution of surface winds has remained approximately constant. Two models of the history of absolute rotation of the Pacific plate are used to determine the past position of GPC-3. The first model²⁵ includes a change in the rotational rate between 50 and 25 Myr ago. The second model²⁶ assumed constant rotation back to 42 Myr ago instead of a slower rotation between 25 and 50 Myr ago.

The sediment in GPC-3 deposited from 65 to 22 Myr ago consists of fish debris, smectite, phillipsite, feldspars and clinoptilolite which corresponds to the authigenic assemblage now found south of 20° N. From about 22 Myr ago to the present, the sediment in GPC-3 consists of manganese micronodules, fish debris, quartz, smectite, chlorite, cristobalite, kaolinite, illite, feldspars, mica and anatase; this corresponds to the detrital assemblage found north of 20° N. Thus, the location of GPC-3 passed through two different sedimentation regions during the past 65 Myr; the transition in the core between the two sediment assemblages occurred between 30 and 20 Myr ago (12–9-m core depth).

The sedimentary transition at 30–20 Myr ago corresponds to a palaeolatitude of 22–24° N using both rotational models, and is in close agreement with the present approximate boundary at 20° N between the authigenic and detrital assemblages. The discrepancy of 2–4° latitude between the present-day boundary and that predicted by the model is not considered significant because of the uncertainties in dating the plate models and the approximate location of the sedimentary boundary. Thus the proposed sedimentation model seems to explain most of the variation observed in the sediment of GPC-3, and indicates that the position of the winds has been relatively stable during the Cenozoic. Our interpretation is limited by the analysis of one giant piston core, but it provides the first attempt to determine a detailed sedimentation history in the red clay region of the central North Pacific.

Cristobalite spheres are found scattered in the lower part of the core (22–24 m) which are not shown in either the X-ray data or the >38-μm sediment analysis due to their intermediate size (our work in preparation). The first plate model²⁵ accounts for this presence of silica, but the second plate model²⁶ predicts that silica would be present higher in the core, unless the band of siliceous sediments now found south of 10° N was further to the south in the early Tertiary. The cristobalite in the top portion of the core in the <20-μm fraction is presumably derived from continental weathering. The distribution of manganese micronodules in GPC-3 cannot be related to the sedimentation model because the present-day distribution is not known. The large abundance of zeolites in the middle of the core is probably derived from dispersed volcanic ash between the individual ash layers.

Two of the six ash layers between 9 and 13.5 m have thin

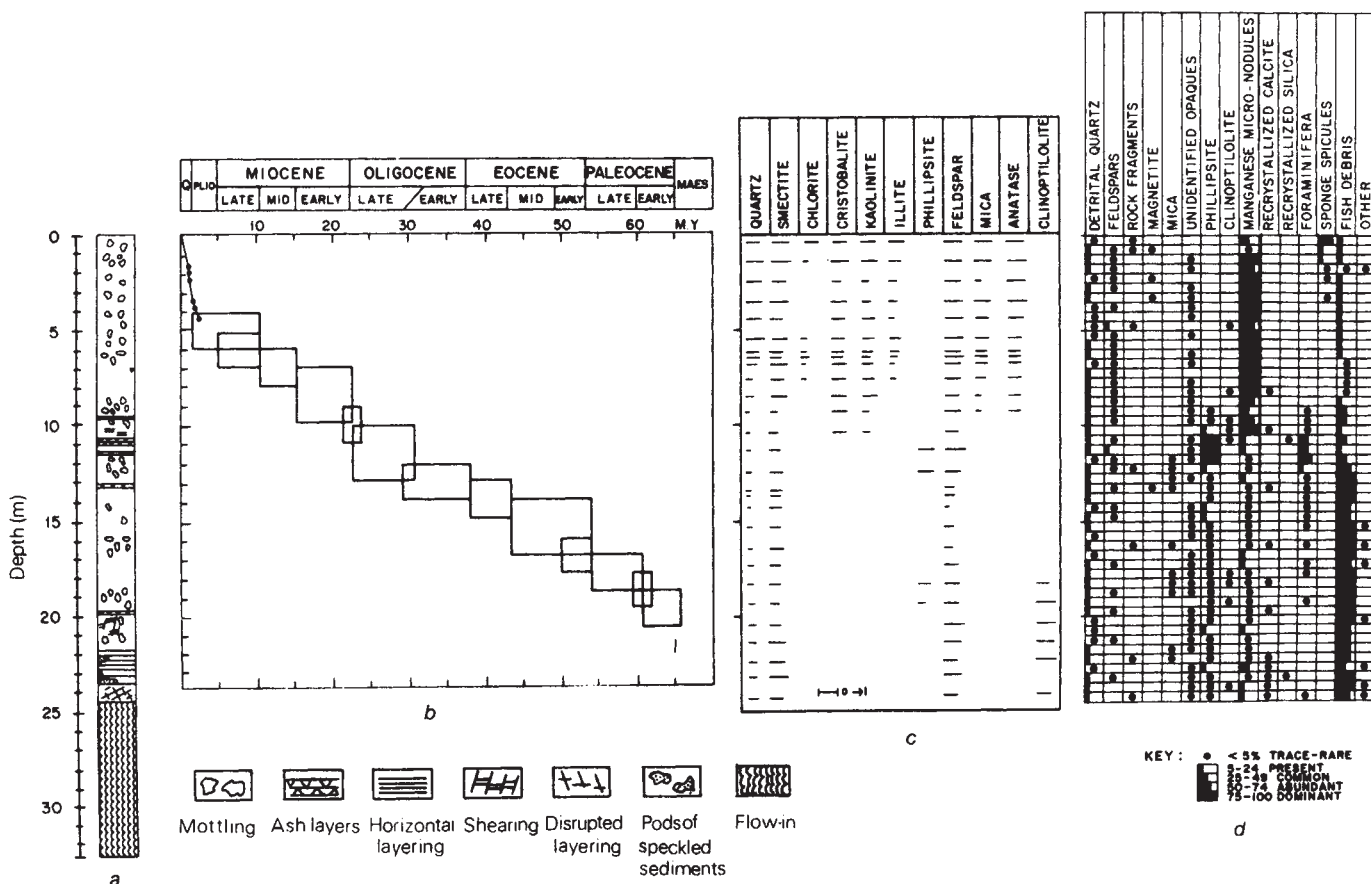


Fig. 1 Summary of lithologic, stratigraphic and sedimentological data in GPC-3. *a*, Lithology. *b*, Age-depth diagram based on palaeomagnetic stratigraphy (Prince *et al.*, in preparation) shown as solid dots, and the ichthyolith stratigraphy (Doyle and Riedel, in preparation) is shown as rectangles. The horizontal lines are the age estimates for each sample, and the vertical lines are the distance between samples, with the sample location at the mid-point. *c*, Mineralogy of the <20-μm fraction determined by X-ray analysis. Normalised abundances are shown and are calculated by dividing the X-ray peak height by the maximum peak height of each individual mineral. *d*, Sediment components of the >38-μm fraction.

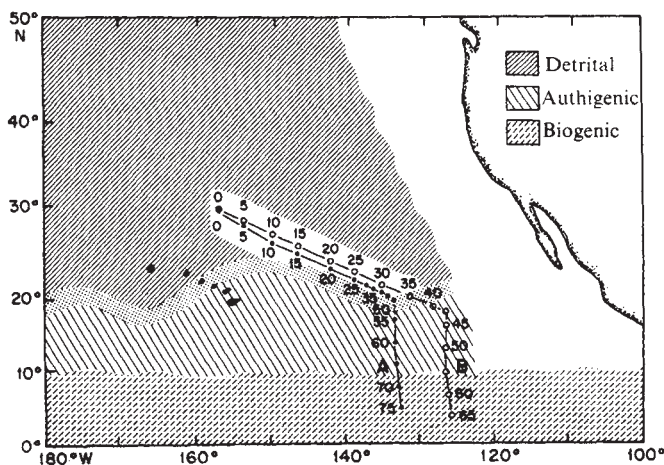


Fig. 2 Migration of the GPC-3 site during the Cenozoic based on the models of van Andel *et al.*²⁵ (A) and Dalrymple *et al.*²⁶ (B) of movement of the Pacific plate. GPC-3 travelled through an authigenic sedimentation region south of approximately 20° N, and a detrital sedimentation region north of 20° N. The boundary between the two assemblages is indicated with dots. The sedimentation patterns bordering the continent are not included, as they are more complex than those found in the central North Pacific.

manganese crusts. The manganese crusts could be explained by a change in Eh of the sediment caused by the ash fall, mobilising the Mn, which then precipitated on the ash layer. The lack of Mn on the remaining ash layers would require the Eh of the sediment to have remained constant during these ashfalls. A second explanation is that the crusts resulted from periods of non-deposition^{27,28}. The Mn layers are thin (0.1 cm or less), indicating that if they were formed during hiatuses caused by bottom currents, these were of short duration.

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Uracil in carbonaceous meteorites

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Previous analyses of carbonaceous chondrites have demonstrated the presence of the biologically significant purines adenine, guanine, hypoxanthine and xanthine¹⁻³. To date, however, no pyrimidine of biological importance has been reported. An earlier report⁴ of a fraction extracted from the Murray meteorite showing 'cytosine-like' spectral characteristics was later shown to be due to an artefact of the analytical method^{5,6}. Some unusual pyrimidines which have no known biological function were reported in extracts from the Murchison, Murray and Orgueil carbonaceous chondrites⁷. However, these results have not been replicated and are now thought to have been due to artefacts^{2,3}. Because of the reported synthesis of the pyrimidines uracil, thymine and cytosine in Fischer-Tropsch-type reactions^{8,9} and the suggestion that such reactions may have been of significance in the production of organic material in meteorites^{9,10}, we have reinvestigated the possible occurrence of pyrimidines in extracts from the Murchison, Murray and Orgueil carbonaceous meteorites using specific fractionation techniques and high-sensitivity analysis. The pyrimidines uracil, thymine and cytosine, together with the purines adenine and guanine are, of course, the building blocks of terrestrial genetic inheritance. Therefore their presence or absence in meteorites is also of considerable interest to theories of chemical evolution and the origin of life. We report here the positive identification of uracil in water and formic acid extracts of all three meteorites.

The meteorite fragments studied were Murchison (6 g, cat. no. 828) and Murray (5.1 g, cat. no. 635.2), obtained from the Center for Meteorite Studies, Arizona State University, Tempe, Arizona, and Orgueil (18.35 g, cat. no. 362), obtained from the Muséum National d'Histoire Naturelle, Paris, France. A 6-g portion of this sample was used for analysis. Allende (5 g) was a portion of a sample described previously³ and was used as a procedural blank.

Pulverised meteorite samples, contained in Pyrex tubes (100 ml) with Teflon-lined screw caps, were extracted twice each with benzene (4 ml g⁻¹) and H₂O (4 ml g⁻¹), and three times each with HCOOH (8 ml g⁻¹) for 30 min at 60 °C in a Sonicator model SC-400 ultrasonic tank. Extracts were recovered by centrifugation and evaporated under reduced pressure at 50 °C. H₂O and HCOOH extracts were desalted and fractionated on charcoal columns as described previously³ and were hydrolysed in 3 M HCl (8 ml) at 110 °C for 18 h. After hydrolysis the solutions were diluted to 1 M HCl, extracted with ether (3 × 25 ml) to remove compounds that are neutral in acidic solution (such as aromatic carboxylic acids), and redesalted. The purified extracts were taken up in H₂O and applied onto BioRad AG50WX8 (H⁺) columns (60 × 4 mm, 50–100 mesh), which had previously been eluted with 5 M HCl and H₂O.

Control experiments using mixtures of the pyrimidines uracil, cytosine, thymine and 4-hydroxypyrimidine and the purines adenine, guanine, hypoxanthine and xanthine had shown that uracil and thymine were selectively eluted from such columns in H₂O, whereas 5 M HCl was required to elute the other bases. The H₂O eluates from the columns were evaporated under reduced pressure at 50 °C and taken up in 0.1 M NH₃ (40 µl).

Analysis was performed by both cation and anion exclusion chromatography using Aminex A25 and A6 columns as described previously³. The complete analysis of other fractions will be reported elsewhere.

UV absorption of the eluates was monitored at 254 and 280 nm simultaneously (see Fig. 1). Peaks in the uracil position

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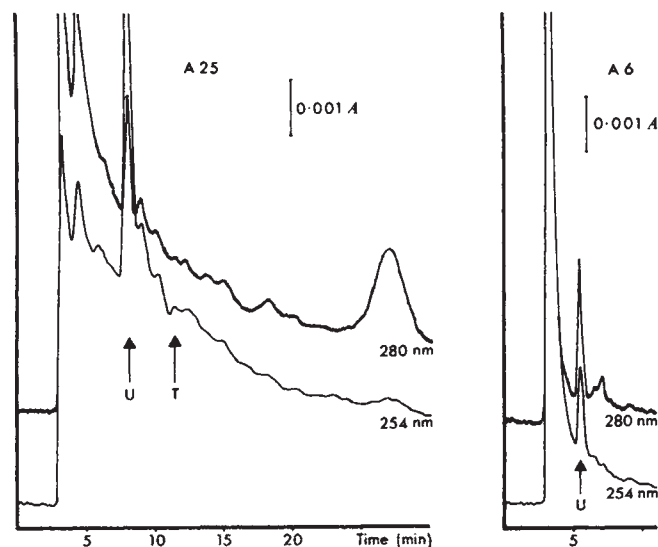


Fig. 1 Chromatograms of the formic acid extract of the Murchison meteorite. A25 shows the fractionation of the purified extract on Aminex A25 at pH 4.0 while A6 is the refractionation of the isolated uracil peak on Aminex A6 at pH 10.0 (see ref. 3 for details). U, Uracil; T, thymine.

of the A25 cation exclusion chromatograms were refractionated on A6 and collected for analysis by mass spectrometry.

Although thymine could not be positively identified, for comparison, maximum concentrations were calculated from peak heights. Initial identification of uracil was based on comparison of retention times and the ratios of the UV absorbance at 280 and 254 nm with those of authentic standards. Retention times on both columns agreed to within 1% or less with those of authentic uracil, while the 280/254 absorbance ratios agreed to within 10% or less. Results are summarised in Table 1.

The identity of uracil was confirmed by programmed ion monitoring on a Finnigan model 3100 mass spectrometer. An intense response for both m/e 112 (M^+ of uracil) and m/e 69 ($M-HNCO$) was obtained from all isolated peaks in the uracil position of both LC columns. In addition, the volatilisation behaviour of all samples matched that of an authentic uracil sample.

Procedural blanks run on extracts of the Allende sample were carried through all stages of the analysis and revealed no measurable contamination with uracil or thymine. Amounts of these pyrimidines corresponding to 0.5 ng per g of meteorite would have been detectable.

Studies of the distribution of biogenic purines and pyrimidines in various soils and recent sediments demonstrate a marked lack of uracil with respect to the other bases¹¹⁻¹³. Reported uracil-to-thymine ratios do not exceed unity and decrease rapidly in older samples^{13,14}. A contemporary zooplankton sample has been shown to have a uracil-to-thymine ratio of 1.75 (ref. 13). In contrast, the Orgueil, Murchison and Murray meteorite samples

analysed in this work show uracil-to-thymine ratios exceeding 15. These results strongly suggest an indigenous origin for the uracil.

It is not surprising that there has been no previous report of uracil occurrence in carbonaceous meteorites, considering its low abundance in the three meteorite samples studied. In analyses of unfractionated meteorite extracts its presence may easily be masked by other more abundant components.

Among compounds synthesised in Fischer-Tropsch-type reactions in conditions resembling those thought to have occurred during the early history of the solar nebula^{9,10}, biologically important amino acids, purines and pyrimidines have been identified^{8,9,15,16}. With the notable exception of pyrimidines, these same compounds have also been reported to exist in carbonaceous meteorites^{1-3,8,17,18}. Although it is unlikely that a single mechanism can explain the patterns of organic compounds observed, the present identification of uracil in the Orgueil, Murchison and Murray meteorites does seem to support the earlier hypothesis^{9,10}, that Fischer-Tropsch-type reactions may have played a part in the synthesis of organic material in carbonaceous chondrites.

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K-Ar dating of the Pleistocene fossil hominid site at Chesowanja, North Kenya

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Reliable radioisotopic dates on igneous material related to fossils and artefacts excavated at a hominid site are valuable to anthropologists who, aided by stratigraphy and palaeomagnetic studies, can then conduct inter-site correlations to erect an overall time scale for hominid evolution in East Africa. As more K-Ar dates become available comparisons become more secure and the time scale more accurate. With this in mind we examined the Chesowanja locality¹ in the northern Rift Valley of Kenya, east of L. Baringo at 36° 12' E, 0° 39' N, and now report a new K-Ar age constraint of $>1.42 \pm 0.07$ Myr for the *Australopithecus* remains and the associated Oldowan tool industry. This minimum was formed by dating the Chesowanja basalt overlying the fossiliferous sediments. Previously, a K-Ar age of 0.73 ± 0.07 Myr had been reported² for this basalt which (1) cast doubt on an age range estimate of 1.37-1.98 Myr for the sediments inferred³ on the basis of a faunal correlation with the K-Ar dated tuffs of the Shungura Formation in the Omo Valley⁴, and (2) confused the interpretation of the geomagnetic reversal stratigraphy⁵.

Table 1 Uracil (U) concentrations and estimated maximum concentrations of thymine* (T) in extracts of carbonaceous meteorites (ng per g sample)

Meteorite	H ₂ O extract		HCOOH extract		Total	
	U	T	U	T	U	T
Allende	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5
Orgueil	46	<4	27	≤1	73	≤5
Murchison	30	<2	33	≤1	63	≤3
Murray	19	≤1	18	≤1	37	≤2

* Thymine concentrations were estimated from observed peak heights in the positions expected for thymine on the chromatograms.

A summary of the stratigraphical sequence is shown in Fig. 1. The top 2 m of the Chemoigut sediments have been baked by the lower flow of the Chesowanja basalt which was exposed long enough to develop a red weathering profile on its surface before being covered by the upper flow. This in its turn was weathered but with a characteristic top of spheroidal boulders which form the base of the 3-m thick palaeosol member. Artefacts of the Acheulian tradition have been discovered in the upper part of this ancient soil horizon.

It was initially believed that this sedimentary sequence was deposited in the Chemoigut basin as a result of a trachyte lava damming the northern outlet. K-Ar dating carried out on this Chepchuk lava placed a maximum age of 1.21 ± 0.04 Myr therefore on the hominid fossils¹. However, the geological relationship of the Chepchuk lava to the section in Fig. 1 cannot be substantiated in the field³, instigating more accurate lines of enquiry. Palaeomagnetic determinations by Brock³ on the baked zone indicate a reversed polarity as do the majority of the Chemoigut sediments⁵. Chesowanja basalt samples yielded a mixture of contrasting polarities but the overlying Karau tuffaceous sediments gave predominantly normal directions. These are succeeded by a sequence of magnetically reversed welded tuffs sandwiching the Karau trachyte dated at 0.55 Myr (ref. 3). The available evidence strongly suggests that the Chemoigut and Chesowanja formations were laid down in the Matuyama Reversed Epoch extending from 2.46 to 0.71 Myr ago. When the late Professor Bill Bishop collected a sample (M30) from the upper flow of the Chesowanja basalt and discovered it had a reversed polarity and a K-Ar apparent age of 0.73 Myr, it was suggested⁵ that the basalt could represent the Brunhes-Matuyama boundary.

A whole rock portion of M30 was sent to Cambridge, crushed to $-45 + 85$ mesh, rinsed in dilute hydrochloric acid, washed and dried. Apart from some alteration of olivine to iddingsite, the rock appeared fresh in thin section. Its potassium content was determined by atomic absorption at 1.006 ± 0.046 (2σ) wt% K_2O , and 6 g were fused and analysed for argon of which 80.28% was atmospheric. Radiogenic argon was present at 4.622×10^{-5} mm³ (STP) per g, which indicates an apparent age of 1.42 ± 0.07 (2σ) Myr. Each age quoted has been calculated according to the constants reported in 1977 (ref. 6).

One of the boulders M30A ($\sim 25 \times 17$ cm) palaeo-weathered from the top of the upper Chesowanja basalt flow was split into three fractions. M30 A X comprised the soft crumbly iron oxide stained exterior, M30 A Y was taken from two, more compact patches of exfoliated exterior, and M30 A Z came from the centre and was therefore least altered. Sample preparation was identical to that for basalt M30 before ~ 0.8 g of each was irradiated for subsequent $^{40}Ar/^{39}Ar$ total fusion analysis^{7,8}. Table 1 summarises the results within analytical error support the more precise conventional K-Ar age determination.

Significant interference from the ^{40}Ca (n, α) ^{36}Ar reaction took place during irradiation⁹. Without correction¹⁰, the apparent age for sample M30 would be 0.55 Myr lower, but for

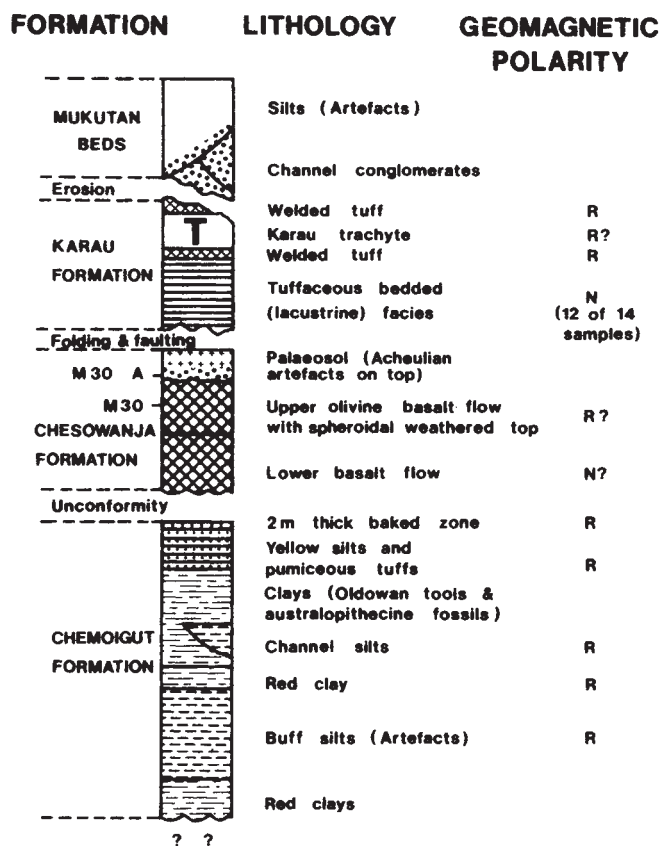


Fig. 1 Simplified stratigraphical section² of the Chesowanja area showing the palaeomagnetic directions⁵ (normal, N; reversed, R).

the weathered and less calcic M30 A samples the ages would differ by 0.3–0.4 Myr. It is interesting how the M30 A determinations give similar results to the analyses carried out on the geologically fresh parent material. The clay minerals formed by the palaeo-weathering process soon after extrusion of the upper basalt flow have largely retained their accumulations of radiogenic argon.

Establishing the apparent age of the Chesowanja Formation at 1.42 ± 0.07 Myr sets a close maximum age for the Acheulian assemblage in the palaeosol member and a minimum date for the australopithecine fossils and non-biface artefacts in the underlying Chemoigut sediments. Using the K-Ar facts the Chepchuk trachyte lava post-dates the hominid finds.

A review of the palaeomagnetic data can now be made which favours a new position for the Chesowanja Formation on the geomagnetic time scale which is before the Jaramillo normal event within the Matuyama Epoch. The Jaramillo event is represented by the tuffaceous facies of the Karau sediments.

Table 1 $^{40}Ar/^{39}Ar$ apparent ages for the Chesowanja Formation

Sample	$J \times 10^{-3}^*$	% Atmospheric contamination	$^{40}Ar_{rad}/^{39}Ar$	Apparent age (Myr)
M30 WR (10 min preheat at $\sim 250^\circ C$)	3.015	92.05	0.2302	1.25 ± 0.26
M30 A X (preheated)	2.963	96.83	0.2564	1.37 ± 0.66
M30 A Y (not preheated)	2.972	96.79	0.2647	1.42 ± 0.70
M30 A Z (preheated)	3.092	92.67	0.2360	1.32 ± 0.42

Errors are 2σ and form a measure of the analytical uncertainties. These are high in view of the large calcium corrections (see text) and the small amounts of radiogenic argon present.

* J is the ^{39}K neutron absorption coefficient derived from the external standard muscovite GG2 (271.7 Myr old).

However, as with the first interpretation⁵, this still leaves a question mark on the anomalously low K-Ar date for the reversely magnetised Karau trachyte, which should lie within the range 0.71–0.91 Myr.

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Relationship of blood lead in women and children to domestic water lead

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Humans can be exposed to lead through food, drink, various occupations or hobbies and also some cosmetics^{1,2}. The contribution of these different exposures to the total body uptake varies between individuals and groups but it is generally accepted that food and drink are the major sources of body lead¹. On average, lead from water contributes markedly less to total intake than does lead from food². It has been suggested, however, that when average lead in water rises above 0.1 mg l^{-1} the intake from water begins to match or exceed that from food². We have previously reported³ that the mean blood lead levels in mothers and children on a housing estate with lead water pipes were over twice as high as those on an adjacent estate using copper pipes. Here we report that the blood lead levels, even when raised, remained very stable when lead exposures were unchanged. The removal of the lead water pipes produced a drop of approximately 50% in mean blood lead levels, reducing them to levels comparable to those on the copper piped estate. No other changes in lead exposure could be found to explain the variations in blood lead levels between the estates, and therefore the possibility of the large contribution of water lead to blood lead should be considered where the conditions described below are known to occur.

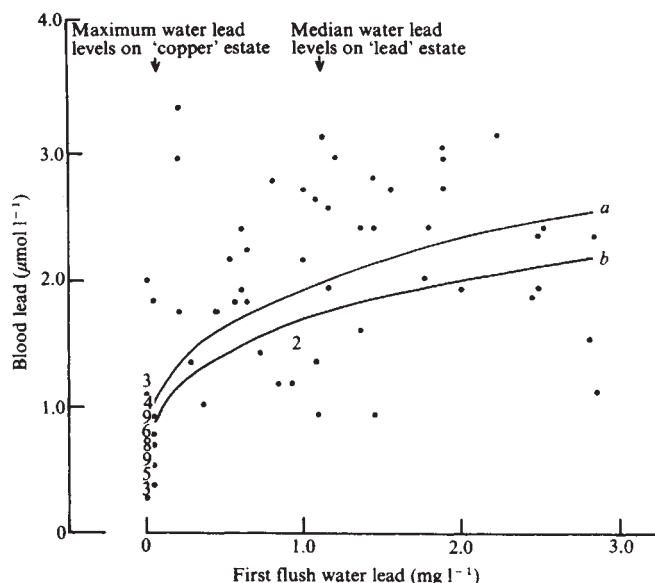


Fig. 1 Relation of blood lead (adult female) to first flush water lead in combined estates. (Numbers are coincidental points; 9 = 9 or more.) Curve a, present data; curve b, data of Moore *et al.*⁷.

The two adjacent council housing estates are situated on the outskirts of a small rural town. The 'lead' estate was built in about 1935 and consists of 60 houses, 55 of which had 4–6 m of external lead piping and 26 of which also had 1–2 m of internal lead piping to the kitchen tap. Lead pipes to the remaining five houses had been replaced with copper before this study. The 'copper' estate comprises 75 houses (built in 1948) and 20 old peoples' maisonettes (built in 1968) and is entirely copper piped. Water for both estates is supplied from a small upland reservoir, and is soft (total hardness $13 \text{ mg l}^{-1} \text{CaCO}_3$), acidic (pH 5.6) and lead free at source.

Water samples (1.2 l polythene bottles; Water Research Centre (WRC) method⁴) were collected from the cold kitchen tap of each house on three occasions at 2-week intervals. The samples were 'daytime' (first water out of the tap at the time of visit), 'running' (after the tap has been running moderately for 5 min after the 'daytime' sample) and 'first flush' (first water out of the tap in the morning before any other water has been run to waste, collected by residents). Samples were acidified (5 ml 1 M HCl) within 12–24 h of collection. Lead estimations were carried out by a Welsh Water Authority (WWA) laboratory that had collaborated with the WRC in an inter-laboratory harmonisation study to establish reliable methods of analysis⁴ and quality control⁵.

Table 1 Relationships between three methods of water sampling and blood lead in 55 adult females and 39 children in the lead estate, and 60 females and 20 children in the copper estate

	Lead estate				Copper estate			
	FF	Water lead (mg l ⁻¹) Day	Run	Blood lead (μmol l ⁻¹)	FF	Water lead (mg l ⁻¹) Day	Run	Blood lead (μmol l ⁻¹)
Adult females								
Minimum	0.002	0.002	0.002	0.9	0.002	0.002	0.002	0.3
Median	1.075	0.568	0.139	1.9	0.004	0.003	0.003	0.7
Maximum	2.826	1.385	0.354	3.3	0.060	0.030	0.018	2.0
Children								
Minimum	0.002	0.002	0.002	1.0	0.002	0.002	0.002	0.4
Median	1.075	0.552	0.147	1.8	0.003	0.003	0.003	0.8
Maximum	2.826	1.276	0.354	3.2	0.060	0.020	0.018	2.2

Water samples were obtained from all dwellings except one which was vacant and therefore excluded. The proportion of eligible subjects providing blood samples: lead estate, adult females 96%, children 100%; copper estate (only two-thirds eligible subjects approached), adult females 95%, children 100%. Reproducibility of blood lead measurements: within laboratory c.v. 8.3% (75 blind duplicates); between laboratories, c.v. 12.4% (Leeds: London; 50 blind duplicates) and 10.2% (Leeds: Glasgow; 18 blind duplicates). Estimate of variation based on 'within sample' variance as distinct from 'between sample' variance. FF, First flush; Day, random daytime sample; Run, after 5 min running.

Blood samples (adult: antecubital vein 2.5 ml into EDTA; young children: capillary) were taken from the adult female residents who spent most time in the house and usually from the youngest child. Lead estimations (including blind duplicates) were made by the NHS Supra-Regional Laboratory at Leeds, with sub-samples being analysed at London (Institute of Child Health) and Glasgow (Department of Materia Medica).

The overall water lead and blood lead levels measured are shown in Table 1. Blood lead levels were markedly higher in the lead estate than in the copper estate. This was true for both adults and children, and has been discussed elsewhere³. No subject had exposure to lead through occupation. Only one woman has a hobby (in this case, enamelling) which involved the use of lead. However, her blood lead level ($1.5 \mu\text{mol l}^{-1}$) was below the median level ($1.9 \mu\text{mol l}^{-1}$) found on the estate on which she lived, suggesting that this hobby did not involve a high exposure to lead.

Detailed analyses (not presented here) of the dependence of blood lead on water lead gave no evidence that any of the water sampling methods—'daytime', 'running' or 'first flush'—was a markedly better predictor of blood lead than the others.

The stability of blood lead within subjects is of interest in this kind of work⁶. We therefore examined this and found that, at least over a period of weeks, blood lead, even when raised, is extremely stable (Table 2).

Table 2 Stability of blood lead ($\mu\text{mol l}^{-1}$) over time in adult female subjects

Time interval	No. of subjects	Mean of first and second sample	Mean change (s.d.)	c.v. (%)
2 weeks	14	1.6	+0.07 (0.21)	9.7
4 weeks	19	1.8	-0.10 (0.17)	6.6
Over 12 weeks	12	0.8	0.00 (0.15)	13.1

No individual was included in more than one subgroup. Coefficients of variation based on 'within subject' variance. These are very similar to the within laboratory c.v. (see Table 1) which suggests that 'true' blood lead is very stable.

Following the removal of the lead pipes, water lead levels became indistinguishable within a few weeks from those on the copper estate. The fall in blood lead (Table 3) was determined in sub-samples of adult subjects examined up to 9 months after the lead pipes had been removed. The mean decrease after pipe removal was approximately 30% at 3 and 4 months and 50% at 6 and 9 months. The blood lead levels had thus become comparable to those on the copper piped estate in approximately 6 months. Changes in blood lead for a small number of controls on the copper estate were also measured and were negligible. No other changes in lead exposure were apparent during this time and therefore the reduction in water lead may be assumed to have produced the fall in mean blood lead levels observed.

Various empirical models (for example, simple linear, quadratic, square root, cube root) were used for regressing blood lead against water lead in our data. The curvilinear models gave a slightly better fit than the simple linear and Fig. 1 shows the regression line of blood lead on the cube root of first flush water lead. This empirical relationship was suggested by Moore and colleagues⁷ from their Scottish data, and the line determined by their regression coefficients is also shown in Fig. 1. The similarity of the curves is interesting as our population sample is quite different from theirs. However, a close similarity between Welsh and Scottish data has also been pointed out previously⁸.

The lead piped estate on which this work was based seems to be exceptional in terms of water lead levels. Over 70% of the houses had first flush water lead levels above 0.3 mg l^{-1} compared with less than 1% of the results for Wales in the survey carried out by the Department of Environment². In conjunction with the WWA, we sampled drinking water from over 20 areas in North Wales considered by water boards to have a possible plumbo-solvency problem but no estate was found with lead levels approaching those reported here. It is therefore probable that we are reporting an exceptional case,

Table 3 Mean blood lead levels ($\mu\text{mol l}^{-1}$) of subgroups of adult females in the lead estate, and the fall in blood lead following removal of the lead pipes

Interval (months)	n*	Initial blood lead (s.d.)	Mean fall after pipe removal (s.d.)	% Mean decrease
3	13	2.2 (0.6)	0.7 (0.3)	32
4	11	1.7 (0.6)	0.6 (0.3)	35
6	10	2.2 (0.5)	1.1 (0.3)	50
9	11	2.3 (0.5)	1.1 (0.3)	48

* No individual included in more than one subgroup.

but water authorities should obviously ensure that no such estates exist within their areas. Where problems exist, removal of lead plumbing is a very effective remedy but other, more economic methods of plumbo-solvency control are being developed⁹.

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Reappraisal of energetics of locomotion shows identical cost in bipeds and quadrupeds including ostrich and horse

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Animals use different amounts of energy to move from place to place depending on their size and mode of locomotion¹. Flyers and swimmers use less energy to move a unit mass a unit distance than do running animals, and small animals use more energy than large ones. For terrestrial animals Taylor *et al.*² have defined cost of transport as the slope of the regression line relating weight-specific metabolic power and running speed. Cost of transport, so defined, is a comparative index of the relative energy-cost of locomotion of different animals. Fedak *et al.*³ had reported that bipeds and quadrupeds have different costs and that, extrapolating from their data, the difference between them would be greatest among large animals. Now, after considering new data from 100-kg ostrich and horse, and reviewing the data collected in the past 5 yr, we find that there are no consistent differences in energy-cost between bipeds and quadrupeds of any size. The apparent differences reported earlier was biased by an unfortunate choice of animals. The new, much more extensive, evidence shows no difference of the scaling of energy requirements for locomotion between bipeds and quadrupeds, but suggests a difference between apparently clumsy and graceful animals.

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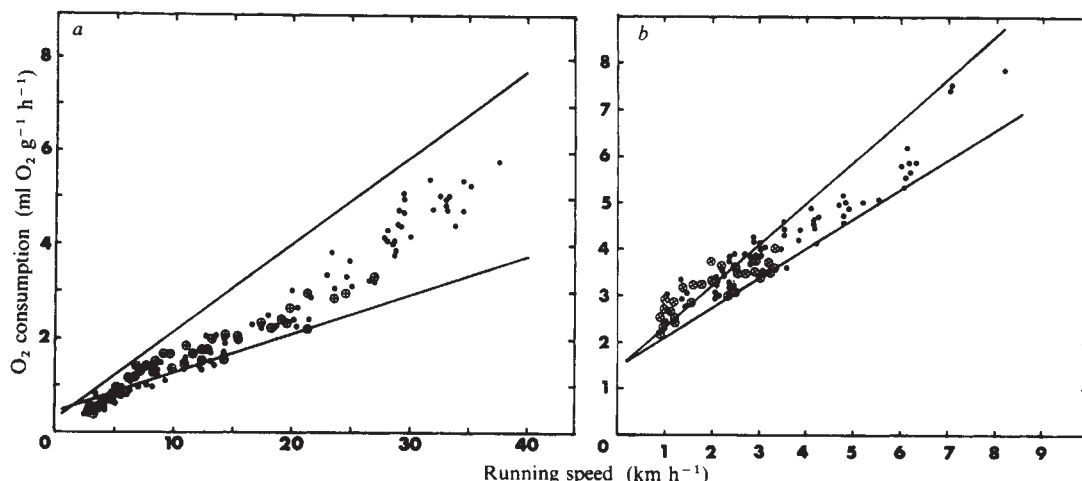


Fig. 1 *a*, The weight-specific oxygen consumption ($\dot{V}_{O_2}$ in $\text{ml O}_2 (\text{g h}^{-1})^{-1}$) of the ostrich ($\oplus$, 103 kg) and horse ($\bullet$, 107 kg) expressed as a function of running speed (km h^{-1}). The upper and lower solid lines are those predicted refs 3 and 2 for 100-kg bipeds and quadrupeds, respectively. Oxygen consumption is a measure of the metabolic power developed by the animal. (To convert $\text{ml O}_2 (\text{g h}^{-1})^{-1}$ to W kg^{-1} multiply by 20.1 W per ml O_2 consumed). We used an open-flow gas analysis system as described in

ref. 3. The large animals ran wearing loose-fitting masks which allowed capture of all expired gas. O_2 and CO_2 concentrations were measured in ambient and post-mask air. Oxygen consumption was calculated by the following formula:

$$\dot{V}_{O_2} = \frac{\dot{V}E(FI_{O_2} - FE_{O_2}) - \dot{V}_{CO_2}}{1 - FI_{O_2}}$$

where $\dot{V}_{O_2}$ is the animal's O_2 consumption; $\dot{V}_{CO_2}$ is CO_2 production; $\dot{V}E$ is flow out of the mask; FI_{O_2} is fractional concentration of O_2 in ambient air; FE_{O_2} is fractional concentration of O_2 in air leaving the mask. The power developed by both animals is nearly identical over the speed range common to both. Regression of $\dot{V}_{O_2}$ on running velocities between 2 and 28 km h^{-1} yields the following equations:

$$\begin{aligned} \text{ostrich: } \dot{V}_{O_2} &= 0.11x + 0.34; & r^2 &= 93\%; & \text{syx} &= 0.20 \\ \text{horse: } \dot{V}_{O_2} &= 0.15x + 0.14; & r^2 &= 96\%; & \text{syx} &= 0.34 \end{aligned}$$

where x = running velocity in km h^{-1} and $\dot{V}_{O_2}$ is in $\text{ml O}_2 (\text{g h}^{-1})^{-1}$.

The slopes of these lines (T_{run}) are used as an index of cost of transport in Fig. 2. The horse data over the entire range of speeds ($2\text{--}38 \text{ km h}^{-1}$) are better fit by a second degree polynomial:

$$\dot{V}_{O_2} = 0.0016x^2 + 0.091x + 0.22; \quad r^2 = 97\%; \quad \text{syx} = 0.31.$$

b, Data as in *a* for two smaller animals: a bipedal bird (road runner, $\oplus$, 285 g) and a quadruped (ground squirrel, $\bullet$, 205 g). The upper line is that predicted for quadrupeds² and the lower is that for bipeds³, both for animals of 245 g. The regression equations for each are:

$$\begin{aligned} \text{road runner: } \dot{V}_{O_2} &= 0.59x + 2.2; & r^2 &= 73\%; & \text{syx} &= 0.26 \\ \text{ground squirrel: } \dot{V}_{O_2} &= 0.64x + 2.0; & r^2 &= 91\%; & \text{syx} &= 0.33. \end{aligned}$$

(On the assumption that 1 ml O_2 consumed yields 20.1 J of energy, then $1 \text{ ml O}_2 \text{ g}^{-1} \text{ h}^{-1} = 5.58 \text{ W kg}^{-1}$.)

Like resting metabolic rate, the energetic cost of locomotion in running animals varies in a regular way with body size. Taylor *et al.*² reported that cost of transport in quadrupeds (T_{run} , expressed in units of $\text{ml O}_2 (\text{g km}^{-1})^{-1}$) decreases as body mass (M) increases, that is, $T_{\text{run}} = 8.5 M^{-0.40}$. Taylor and Roundtree reported no difference in cost between two species of monkeys (3 and 17 kg) trained to run on both two and four legs. However, Fedak *et al.*³ reported that bipeds in general showed a different allometric relationship with body mass ($T_{\text{run}} = 3.1 M^{-0.24}$). Because of the relative magnitude of the two constants, the predictions for the metabolic power requirements were nearly identical for animals of 1 kg. However, small bipeds seemed to have a lower cost of transport than small quadrupeds. For example, for a 40-g animal, the predicted cost for a quadruped is 1.5 times that for a biped. Among large animals however, bipeds, not quadrupeds, seemed to face much larger energy costs. For a biped the size of *Aepeornis* (450 kg) the cost would be almost 3 times as high as for a similar-sized quadruped. If this difference in energy cost is extrapolated to animals the size of the largest bipedal dinosaurs (about 5,000 kg for *Tyrannosaurus*) the energy cost for bipeds would be more than 4 times that for quadrupeds. Palaeontologists have suggested, on morphological grounds, that some of the large bipedal dinosaurs were fast-moving cursors⁵. Extrapolations of energy-cost from these previously available data suggested either that the morphological evidence is misleading and these animals had low sustained speeds or that large bipeds had very high metabolic scopes and the means to dissipate heat very rapidly.

The data on which the differences between bipedal and quadrupedal locomotion were based were limited, particularly for larger animals. The largest quadruped for which data were available over a wide range of speed was the dog. Humans were the largest bipeds represented. We decided to make a direct comparison of the largest extant biped (the ostrich, *Struthio camelus*) with a quadruped of equal size (a small horse) to see if the predicted difference in cost of transport indeed existed. The prediction would be that the slope of the metabolic power versus speed curve for the ostrich would be twice that of the horse. This difference would be measured easily. In addition we present new data on a small bipedal bird, the road-runner (*Geococcyx californianus*, 285 g), and a quadrupedal ground squirrel (*Citellus tridecemlineatus*, 205 g).

A 103-kg female ostrich and two Shetland ponies (mean body mass, 107 kg) were trained to run on a motorised treadmill while we measured oxygen consumption using an open-circuit gas analysis system. The measurement system and calculations have been described previously (Fig. 1, ref. 3). Both animals ran on the same treadmill and their oxygen consumption was measured using the same system and identical methods during the same 2-month period. All data are for runs of 5 min or longer. The data for oxygen consumption are values below the animal's maximum oxygen consumption and represent the entire metabolic power developed by each of the animals for the ranges of speed for which data are included. The ostrich would run steadily for 5 min or longer at speeds of $3\text{--}28 \text{ km h}^{-1}$. The bird could run much faster for brief bursts but would not sustain

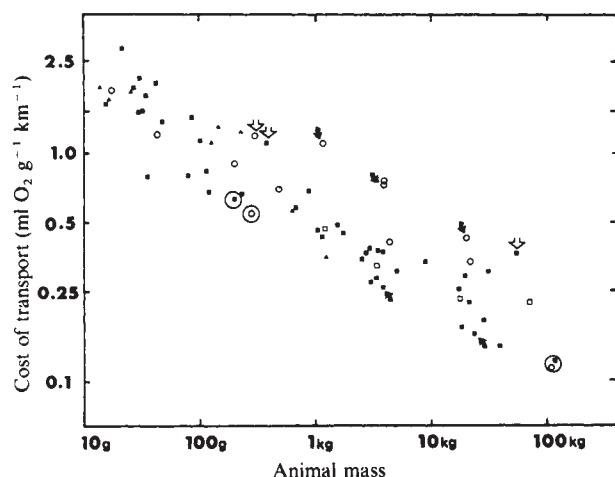


Fig. 2 T_{run} , an index of energy cost of running, plotted as a function of body size for 69 kinds of lizards (Δ), birds ($\circ$) and mammals ($\square$, $\blacksquare$). Bipeds are represented by open symbols and quadrupeds by solid symbols. The symbols for the road runner, ground squirrel, ostrich and horse are surrounded by circles. Solid arrows point to T_{run} data for penguins and geese at the top of the distribution and for dogs at the bottom of the distribution. Open arrows indicate the data for the white rat, tinamou and lion in order of increasing size. The most awkward-looking runners such as geese and penguins show the highest T_{run} for their size while fleet animals such as ground squirrels, dogs and horses, and the ostrich, show a low T_{run} . The slopes of the least square regression lines for bipeds and quadrupeds are not significantly different ($T = 0.2$ for $69 - 2$ degrees of freedom). The equation and regression statistics for the pooled data follow: $T_{\text{run}} = 3.89M^{-0.28}$. For the log-transformed data: $r^2 = 84\%$ (adjusted for 67 d.f.); $\text{syx} = 0.14$; s.d. of intercept = 0.05; s.d. of slope = 0.015. The data in this figure come from the following sources.

Animal	Weight (kg)	Ref.
Mammals (7)	2–18	2.
Birds (7)	0.04–4	3
Capuchin	3.3	4
Chimpanzee	17.5	4
Lizards (8)	0.014–1.2	5
Mammals (5)	0.2–100	6
Marsupial	0.030	7
Rodent	0.027	7
Marsupials	0.015–1.1	8
Wallaby	3	9
Quakka	3	9
Lion	30	10
Lion	55	10
'Primitive' mammals (5)	0.12–5	11
Hopping mouse	0.037	12
Echidnas (2)	1.5–3.5	13
Human	70	14
Primates (3)	1–4	15
Penguins (3)	1–21	16
Mouse	0.030	17
Chimpanzee	17	17
Rhea	20	18
Mammals (3)	28–44	19
Dog	3	20
African hunting dog	9	20
Squirrel	0.086	21
Squirrel	0.079	22
Capuchin*	4	—
Plovers†	0.018	—

* Personal communication from S. A. Mahoney.

† Personal communication from P. Nicolayasen.

Oxygen consumption accounts for all the metabolic power developed by an animal if there is no steady increase in whole-animal lactate even if individual muscles are producing lactate. We used blood lactate concentration as an index of whole-animal lactate content. Blood lactate in the ostrich never increased above 5 mM at the highest speeds we observed on the treadmill. Higher blood lactate levels (15 mM) could be measured if we caused the animal to run in its pasture towing the authors by a harness. The horse did not show continuously increasing blood lactate until it reached speeds of 38 km h⁻¹. At speeds above this, blood lactate increased continuously to levels of greater than 30 mM and oxygen consumption no longer increased. Details of horse blood lactate and maximum oxygen consumption can be found in ref. 6.

Similar methods were used to measure the oxygen consumption of the smaller animals (three ground squirrels and two road runners) except that the animals were run in ventilated chambers rather than wearing masks.

Figure 1a is a plot of the oxygen consumption of both the ostrich and the horses as a function of running speed. Dotted lines indicate values predicted by the equations currently presented in the literature for bipeds and quadrupeds of this size^{2,3}. The relationship between metabolic power and speed was the same for both animals over the range of speeds they both would run. From 3 to 28 km h⁻¹ the increase in power input with increasing speed is approximately linear. Data from both animals show an inflection in the function when they change from a walk to a run or trot. There is no obvious inflection in the power versus speed relationship when the horse changes from a trot to a gallop (at 17 km h⁻¹).

When the horses ran faster than 28 km h⁻¹, an increase in the rate of change of oxygen consumption with speed became apparent. Most animals, including the ostrich, cannot easily be trained to sustain the high oxygen consumption necessary to establish whether or not this is a general phenomenon. The value of T_{run} we used in Fig. 2 was always the slope of a straight line fitted to the metabolism data. In the case of a horse we only used the data up to 28 km h⁻¹. In all other animals the regression line was fitted to data from the entire range of speeds.

Regression lines relating predicted and observed metabolic power and running speed for two species of small bipeds and quadrupeds (road runner and ground squirrel) are presented in Fig. 1b. Metabolic power increases much more rapidly with speed in these small animals but again there is no difference between the slopes of the regression lines for the quadruped and the biped.

Comparison of all the energy cost of locomotion data now available for a wide variety of animals representing 66 species shows no consistent differences between cost for bipeds and quadrupeds of any size (Fig. 2). This figure includes cost data from reptiles, mammals and birds for various styles of terrestrial locomotion (sources of data in ref. 7). We include all animals for which we could find data over a speed range wide enough to determine a slope. For any given size animal, energy-cost may vary by a factor of nearly two. It is tempting to conclude that animals we tend to think of as cursorial, fast or graceful seem to fall near the lower end of the distribution for their size (that is horses and dogs) while animals we think of as awkward tend to fall high in the distribution (that is, geese and penguins) but this is only a subjective *a posteriori* impression. There are numerous animals represented which show similar costs with quite different styles of locomotion and vice versa (for example, compare the points for the white rat, tinamou and lion indicated by open arrows in Fig. 2). Note that the variation in cost for a given size is small compared to the over 15-fold variation seen between large and small animals. The decrease in energy cost of locomotion with increasing size first reported by Taylor *et al.*² is generally true for all types of terrestrial animal studied regardless of locomotory mode or taxonomic group and is thus of very general predictive value.

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higher speeds long enough to get reliable metabolism measurements. The horse would run steadily at sustained speeds of 38 km h⁻¹ without exceeding its maximum oxygen consumption as indicated by steady low blood-lactate concentrations.

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Gut-associated microflora of *Limnoria tripunctata* in marine creosote-treated wood pilings

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The marine isopod *Limnoria tripunctata* is unique among wood borers in its ability to inhabit and severely damage creosote-treated wooden structures, yet little is known of the nature of this apparent resistance to creosote. When the animal is reared on untreated wood, its digestive tract appears to be free of microorganisms^{2–4}. In contrast, we report here that microorganisms are readily observed in the digestive tract of *L. tripunctata* inhabiting creosote-preserved wooden pilings. Furthermore, isopods from such preserved wood apparently possess a resident gut microflora, which is in close association with the lining of the intestinal tract and is separated by a peritrophic membrane from other microorganisms ingested during wood boring.

Samples of *L. tripunctata* were collected at the US Naval Base at Roosevelt Roads, Puerto Rico in December 1976 and October 1977, from creosote-treated wooden pilings severely damaged by these wood borers. Specimens were preserved immediately and examined later by light and electron microscopy (Fig. 1).

The digestive system of *L. tripunctata* consists of an alimentary tract (mouth, gastric mill, tubular intestine and anus), and two bilobed diverticula which join the alimentary tract just behind the gastric mill⁷. Ingested wood and associated microorganisms were found in the alimentary tracts of *L. tripunctata* specimens examined; however, no ingested material or microorganisms were observed in the digestive diverticula. Gut contents were present in 12 of the 13 specimens examined, and consisted of minute pieces of wood and various prokaryotic microorganisms concluded to be bacteria (Fig. 1a). These bacteria attached by capsular material (that is, polysaccharides stained by ruthenium red) are presumed to represent the

microflora growing on the surfaces of the creosote-treated wood of the pilings and isopod tunnels. Occasionally bacterial cell debris was observed; however, most of the gut microorganisms were intact and some cells were located in pits in the ingested wood (Fig. 1a, arrow). In contrast to wood fragments, ingested bacteria were not uniformly present throughout the gut, suggesting that their presence depended on whether the isopod had been feeding on superficial layers of wood that had been colonised by microorganisms or on the underlying layers of wood not subjected to microbial attack.

Reports of cellulase activity in the digestive diverticula of *L. tripunctata*^{8,9}, coupled with the absence of microorganisms in the digestive tract of isopods reared on unpreserved wood^{2–6}, indicate that *L. tripunctata* can derive nutrition from ingested wood without the aid of microorganisms. Wood is a nitrogen-poor food source and several investigators have suggested that ingested microorganisms supplement the diet of this isopod by providing an additional nitrogen source, essential amino acids and possibly other micronutrients^{2,10}. However, there have been conflicting reports as to the ability of *Limnoria* to attack sterilised wood^{11–14}. Our finding that numerous microorganisms are ingested by *L. tripunctata* during wood boring offers some support for a microbial role in the nutrition of this isopod.

The gut contents, including bacteria, were encased in a peritrophic membrane isolating the ingested material from the intestinal lining (Fig. 1a). The peritrophic membrane of arthropods, which is composed of chitin and protein, is thought to act as a barrier to mechanical abrasion, as well as microbial infection, of the gut epithelium^{15–17}. This same membrane covers egested faecal pellets and may delay microbial attack of

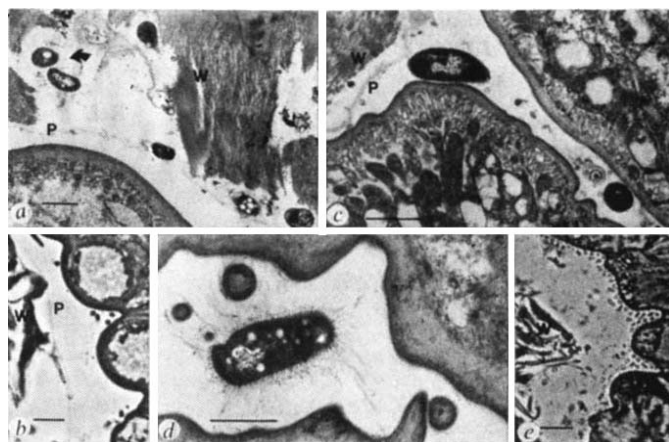


Fig. 1 Sections through the intestinal tract of *L. tripunctata* from creosote-treated wooden pilings at Roosevelt Roads, Puerto Rico. After fixation at the time of collection in a 1% glutaraldehyde solution in sterile seawater with 500 p.p.m. ruthenium red for 2–4 d at 4 °C, whole specimens or dissected digestive tracts were fixed for a further 3 h at 25 °C in 1% osmium tetroxide (0.1 M sodium cacodylate, pH 7.4), then dehydrated (acetone series) and embedded in a known orientation in Epon 812 epoxy plastic. Sections (1–5 µm) were collected, stained with 0.25% safranin (1 min, 60 °C) and examined by phase contrast light microscopy (b, e). In areas of interest thin sections (50–70 nm) were collected, stained with uranyl acetate and lead citrate and examined by electron microscopy (a, c and d). a, Ingested wood fragments (W) and associated bacteria separated from intestinal epithelium by the peritrophic membrane (P). Arrow denotes pit apparently formed by microbial wood digestion (scale bar, 1 µm). b, Gut-associated bacteria, in invaginations of the intestinal tract. Peritrophic membrane indicates border of gut contents (scale bar, 10 µm). c, Gut-associated bacteria in an invagination of the intestinal lining (scale bar, 1 µm). d, Gut-associated bacterium with electron transparent cytoplasmic inclusions and capsular fibrils; longer capsular fibrils connect to an intestinal microvillus in the gut invagination (scale bar, 1 µm). e, Abundant gut-associated bacteria lining intestinal wall and attached to periphery of gut contents in specimen in which peritrophic membrane was not completely intact (scale bar, 10 µm).

the faecal material¹⁸. In *L. tripunctata*, the peritrophic membrane may also function in this manner, in part explaining the lack of microorganisms in the intestinal tract of isopods reared on unpreserved wood and the reported delay in microbial attack of faecal material egested by *L. tripunctata*⁴.

Bacteria were observed in the peritrophic space (that is, the space between the peritrophic membrane and the intestinal lining) of all *L. tripunctata* specimens from creosote-treated pilings. (Fig. 1b, c and d.) These bacteria, which apparently bypassed the peritrophic membrane barrier, are, in effect, separated from the ingested microorganisms and are concluded to reside in the intestinal tract of the isopod, in close association with the intestinal lining. This conclusion is supported by the presence of gut-associated bacteria in the intestinal invaginations of an isopod that did not contain ingested material or a peritrophic membrane (Fig. 1d). The most common type of bacterium, found associated with the intestinal lining, contained numerous electron transparent cytoplasmic inclusions and exhibited capsular material that included small, spike-like projections, and less numerous, larger, fibrillar projections anchoring the cell to the gut surface (Fig. 1d). Occasionally a second type of gut-associated bacterium was observed, which, although close to the intestinal wall, did not possess discernable attachment structures (Fig. 1c). The gut-associated bacteria were present throughout the intestine, generally in small numbers (one to five cell profiles per thin section), with an exception noted in one specimen in which the peritrophic membrane was not completely intact. In this case, the dominant cell type (Fig. 1d) was observed to be present in much greater abundance, with most cells attaching to the gut walls and some cells attaching to the periphery of the gut contents (Fig. 1e).

Kalnins's transmission electron microscopic study of *L. tripunctata* inhabiting untreated pine wood revealed wood fragments, but no microorganisms in the digestive tracts². We have confirmed these results in studies of *L. tripunctata* reared on untreated wood in aquaria, and have also been unable to observe morphological differences between the digestive tracts of isopods reared on untreated wood and those of isopods from creosote-treated wood (our manuscript in preparation). Thus, the gut-associated bacteria we observed do not seem to be pathogenic or opportunistic invaders of *L. tripunctata*.

Electron transparent inclusions, such as those characteristicly observed in the dominant gut-associated bacterium in *L. tripunctata*, accumulate in bacteria which store poly-β-hydroxy butyrate¹⁹, and in bacteria grown in hydrocarbons²⁰. If the latter situation can be demonstrated (that is, the inclusions in the gut-associated bacteria contain hydrocarbons) it is possible that bacterial uptake and/or utilisation of hydrocarbons from ingested creosote contributes to the creosote resistance attributed to *L. tripunctata*.

In conclusion, the discovery of a gut microflora as a unique characteristic of *L. tripunctata* inhabiting creosote-treated wood pilings is of great interest, especially if acquisition of an intestinal microflora contributes to the success of this isopod species, which causes severe damage to creosote-preserved wood structures in Puerto Rican and other tropical waters.

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Regular steps in bending cilia during the effective stroke

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A ciliary beat cycle consists of an effective stroke in which the extended cilium makes an oar-like movement towards one side, and a recovery stroke in which the cilium moves back by propagating a bend from base to tip (Fig. 1A). In the sliding microtubule model of ciliary and flagellar movement, which is now supported by substantial evidence¹⁻³, the sliding displacement of microtubules in any region of the cilium is related linearly to the angular change in the direction of that region (Fig. 1B). Thus, during the effective stroke, microtubule sliding is not confined to the region near the base of the cilium but involves the whole length of the extended organelle, and the relative speed of sliding can be measured as the angular velocity of the ciliary motion. I report here that in molluscan cilia the effective stroke consists of regularly alternating rapid and slow phases of angular movement. This suggests that the microtubules slide in quantal steps.

The movement of the giant compound cilia on the abfrontal surface of the gill of the mussel, *Mytilus edulis*, was photo-

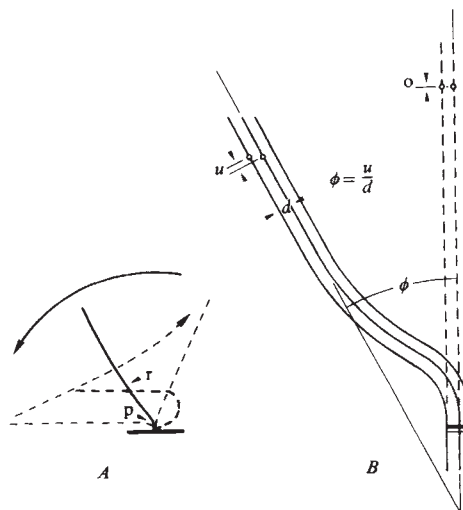


Fig. 1 Schematic diagram of ciliary beat and microtubule sliding in a bent cilium. **A**, A ciliary beat consists of an effective stroke (thick curve with an arrow indicating the direction of movement) and a recovery stroke (thick dashed curve). p and r indicate principal and reverse bends, respectively. **B**, The relative sliding displacement between inextensible microtubules (three parallel curves) related to the angular direction of a cilium. The relationship is expressed by the equation, $\phi = u/d$, where u is the amount of sliding in a segment of cilium (dots), ϕ the angular direction in rad of the segment with respect to the basal region, and d the distance between the tubules measured in the direction parallel to the bend plane.

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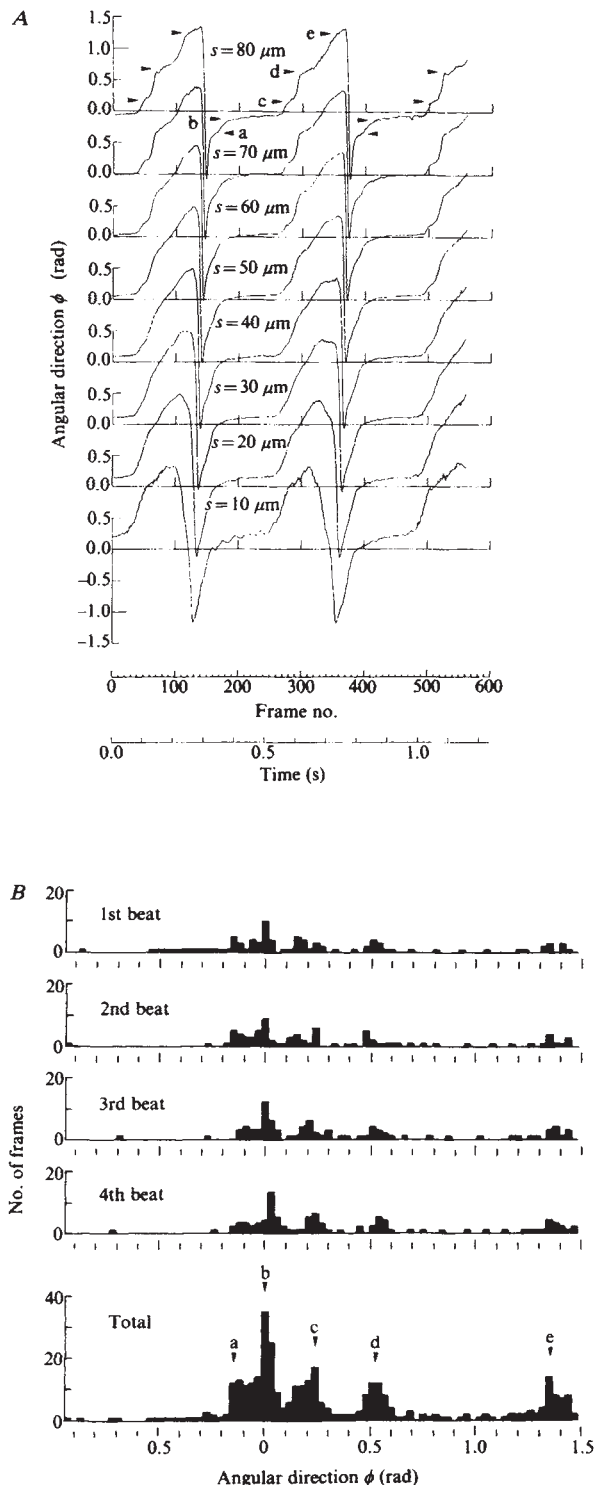


Fig. 2 Changes in angular direction (for definition, see Fig. 1) of large abfrontal cilia of *Mytilus* gill. **A**, The angle is plotted against time for points at 10- μm intervals along a cilium (s , the distance from base as measured along the cilium). Arrowheads a, b, c, d and e indicate slow phases in the effective stroke. The cilium was 90 μm long, and the temperature was 23.5°C. **B**, Histogram of angular direction of the distal part of a cilium during the effective stroke. From a sequence of film (270 frames per s), the frames in which the angular direction was within each successive range of 0.03-rad widths were counted over four successive effective strokes. The cilium was 79 μm long. The beat frequency was 2.1 Hz and the temperature was 24°C. The graphs were made from frame-by-frame analysis⁸ of high speed cinematographs with the aid of a computer at the Computer Centre of the University of Tokyo.

graphed at about 500 frames per s with a high-speed ciné camera. These cilia are suitable for cinematographic analysis because of their large size, sparse occurrence, relatively slow movement and nearly planar beat form⁴⁻⁷. Projection of the film at reduced speed showed the effective stroke to be interrupted, usually five times, by slow phases of various duration. The longest of these (indicated by arrow b in Fig. 2) occurred when the cilium was upright and corresponded to the 'interkinetic pause' described by Gray⁴. Other slow phases were less obvious but could be recognised, particularly in the most distal part of the cilium, by careful watching of the film. In the curve of angle versus time, obtained by frame-by-frame analysis⁸, the slow phases appeared as distinct steps (Fig. 2A). They appeared as peaks in the histogram made of the occurrence of the slow phases over four successive beats (Fig. 2B). They occurred consistently at angles (in rad) of ~ -0.2 ; 0; 0.2–0.3; 0.5–0.7, and 1.3–1.5, regardless of the length of the cilia which ranged from 57 to 90 μm .

As the rapid angular change starts earlier in the proximal region than in the more distal region, there are two bends (Fig. 1A) present on the cilium during the effective stroke, with the proximal one (principal bend) being sharper than the distal one (reverse bend)⁵. Both bends are due principally to the internal force of the cilium^{5,9}. The principal bend grew continuously during the effective stroke, while the reverse bend developed and propagated repeatedly, giving rise to the rapid movements of the distal region.

Similar bends could be induced by applying a force with a microneedle⁹ to the middle portion of the ciliary shaft during the slow phase b (Fig. 3). The induced bend grew and propagated even while the applied force decreased and was finally removed. Thus, bending is an active response to the mechanical stimulus. When the induced principal bend propagated, the distal part of the cilium changed its direction with a step of 1.6 rad (Fig. 3H).

These observations indicate that the microtubules slide with intermittent movements and, because the connecting force between the individual component cilia does not seem to be large enough to retard the movement of the components by itself⁹, this feature of tubule sliding seems to originate in the behaviour of axonemal substructures¹⁰. I propose a model to account for the abrupt changes in sliding speed as follows. The speed of sliding is presumably determined by the force generated by the dynein arms and the resistance to sliding. When the force is generated in the proximal region, the tubules in this region slide to form the principal bend close to the base. On the other hand, the dynein arms in the distal region are not active enough to overcome the resistance to sliding, as indicated by the fact that the angular direction of this region changes little at this stage (slow phase), giving rise to the reverse bend. As the force generated by the arms plus the elastic force of the bent tubules overcomes the resistance in the distal region, the sliding propagates towards the tip (rapid phase). This releases the energy stored in the elastic elements so that sliding in the distal region tends to cease again (second slow phase). It should be noted that the dynein arms and the radial spokes on the sliding doublet change position relative to their sites of interaction on the adjacent doublet and the central tubules, respectively (Fig. 4). This may impose a periodic regulation on force generation and resistance. The arms on doublet no. 3 and the spokes on doublet no. 1 are most efficient in producing force and resistance, respectively, due to their position relative to the bend plane (Fig. 4B). These arms and spokes are thought to pass through one cycle of interaction when they slide by 22.5 nm (ref. 11) and by 28.6 nm (ref. 12), respectively. Calculations showed that the segment of axoneme should change its direction by 0.32 rad for the spokes on doublet no. 1 to complete one cycle of interaction by tubule sliding (Fig. 4A and ref. 12), and 0.36 rad for the arms on doublet no. 3 to complete their cycle (Fig. 4C). Similar calculations gave angles (in rad) 0.32, 0.37, 0.45, 0.51, 0.75, 1.06, 6.36 for the spokes on doublets nos 5, 6, 2, 9, 7, 4, 3, 8, and angles 0.38, 0.40, 0.45, 0.52, 0.64, 0.85 for the arms on doublets nos 7, 8, 2, 4, 6, 9, respectively. In the absence of

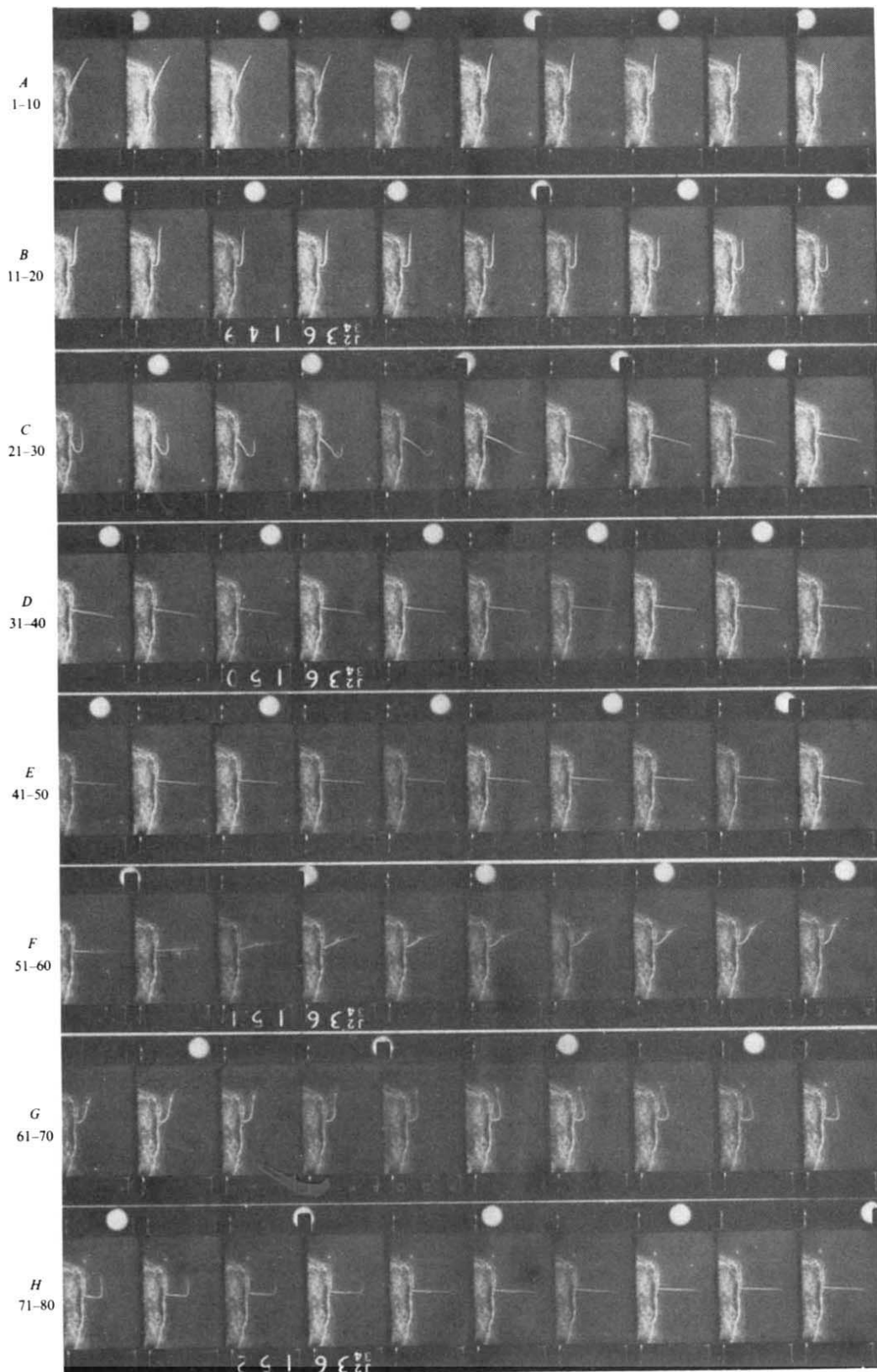


Fig. 3 Normal bending wave during the recovery stroke of a large abfrontal cilium of *Mytilus* gill, and bending wave induced by mechanical stimulation. The cilium was hit by a glass needle (small bright spot) as described previously⁹, in the course of a normal beat, a part of which is shown from the late stage of the effective stroke (A) to the instance of a hit (53 in F) in slow phase b (see Fig. 2). At first the cilium was bent proximal to the needle according to the instantaneous flexural rigidity⁹ and was bent in the reverse direction at the point of contact of the needle (53 and 54 in F). Next, the proximal (principal) bend grew and the reverse bend propagated distally, while the force exerted by the needle decreased (55–61). Then the principal bend propagated along the cilium (62–75) at the same speed as the bend propagation in the recovery stroke (B and C). Finally the cilium was restored to the position of slow phase b (76–80). Note that throughout the response the basal region did not change direction and also that, when the principal bend propagated, the distal region was pointing in the same direction as during the recovery stroke, that is, the direction of the region in slow phase e (Fig. 2). The framing speed was calibrated by bright spots marked at 100 Hz on the upper margin of film. The cilium was 71 μm long, and the temperature was 24 °C.

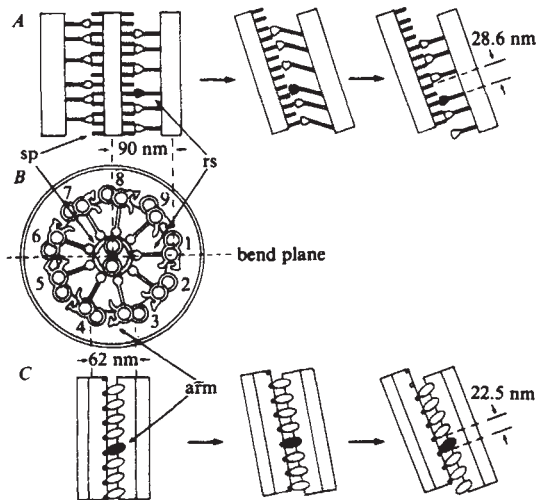


Fig. 4 Hypothetical changes in axonal substructures in a bent cilium. **A**, Doublet no. 1 (see **B**) in a segment of the cilium slides by 28.6 nm relative to the central tubules to complete one cycle of attachment-detachment between the radial spokes (rs) and the central sheath projections (sp)¹². This causes the segment to change its direction by 0.32 rad. The vertical distance between the neutral lines of doublet no. 1 and the central tubules is 90 nm. **B**, A cross section of the cilium. **C**, Doublet no. 3 (see **B**) in the segment slides by 22.5 nm relative to doublet no. 4 to complete one cycle of attachment-detachment between the dynein arms (arm) of doublet no. 3 and the binding sites on the b-tubule of doublet no. 4 (ref. 11). This causes the segment to change direction by 0.36 rad. The vertical distance of the neutral lines of doublets nos 3 and 4 is 62 nm when measured in the direction of one bend plane. The changes in angular direction of the segment mentioned above are calculated by the equation: $\phi = u/d$ (see Fig. 1B). Dimensions are based on values reported by other authors¹⁰⁻¹².

detailed information about the relative positions of arms, spokes and their respective binding sites among different tubules, it is not fruitful to speculate further on this matter. However, it is worth noting that the theoretical minimum angle (for example, 0.32 rad for spokes) approximately matches the observed minimum angular step (0.2–0.3 rad). Further investigations, with single cilia such as those of sea urchin embryos¹³, seem necessary to clarify whether the bend steps in the *Mytilus* compound cilia result from an inherent property of the axoneme rather than from interactions of many component cilia.

A preliminary report of this work has been presented elsewhere¹⁴. I thank Professor K. Takahashi for reading the manuscript and Professors Y. Hiramoto and P. Satir for discussion.

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Non-retinotopic arrangement of fibres in cat optic nerve

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Fibres in the mammalian optic nerve are generally thought to be organised retinotopically. Recording electrophysiologically from the cat optic nerve, we found little evidence to support this notion, which led us to investigate the problem by anatomical methods. We made a localised injection of horseradish peroxidase into the lateral geniculate body of the cat, labelling a small clump of retinal ganglion cells and their axons in the optic nerve. These fibres, emanating from neighbouring cells in the retina, became widely scattered through the optic nerve, indicating that retinotopic order is essentially lacking.

Perhaps no single fibre tract of the central nervous system has been so well studied as the optic nerve pathway from the eye to the brain. Nevertheless, the developmental mechanisms responsible for guiding incoming retinal ganglion cell axons to appropriate sites in their target nuclei, to achieve a precise topographical representation of the visual world, remain poorly understood. One theory, generally known as the 'chemo-affinity theory', states that each axon is attracted by a specific chemical signal to make its proper synaptic connection¹. This proposal does not require retinotopic order in the optic nerve. An alternative theory suggests that fibres leave the retina in a strict retinotopic array and maintain their parallel alignment throughout the optic pathway, to be channelled to correct terminations by simple mechanical forces². This explanation implies that information concerning specificity is embodied in the spatial arrangement of the fibres in the optic nerve and is thus predicated on the assumption that they are organised retinotopically.

The classical neuroanatomical literature is replete with studies reporting that fibres in the mammalian optic nerve are arranged retinotopically (see ref. 3 for summary and further references). These experiments involved making retinal lesions and looking for the pattern of subsequent degeneration in the optic nerve. Results obtained by this technique were difficult to interpret because the retinal lesion unavoidably damaged optic fibres passing across the retinal surface from more peripheral regions, and the degeneration stains were often unreliable. A few investigators actually failed to find evidence favouring retinotopy, but these negative reports received little attention^{4,5}. The evidence available from physiological recordings made 20 yr ago in the spider monkey optic nerve suggested a scattered distribution of receptive fields⁶. Given this uncertainty over an issue of such importance to theories of neural development, it seemed worthwhile to re-examine the question of retinotopy in the mammalian optic nerve by physiological methods and modern anatomical techniques.

We began by repeating the physiological experiments in the cat. Recordings were made from three adult cats under thiopental anaesthesia according to standard procedures⁶. A tungsten microelectrode was passed vertically through the optic nerve at the point where it emerges from the optic canal to enter the cranial cavity (Horsley-Clarke V –4.00, A 15.75, L 3.75), and the location of each receptive field encountered was mapped in the animal's visual field. Our findings essentially confirmed the previous spider monkey study. In a typical penetration (Fig. 1) receptive fields of successively recorded fibres were located in widely separated areas of the visual field. Little hint of retino-

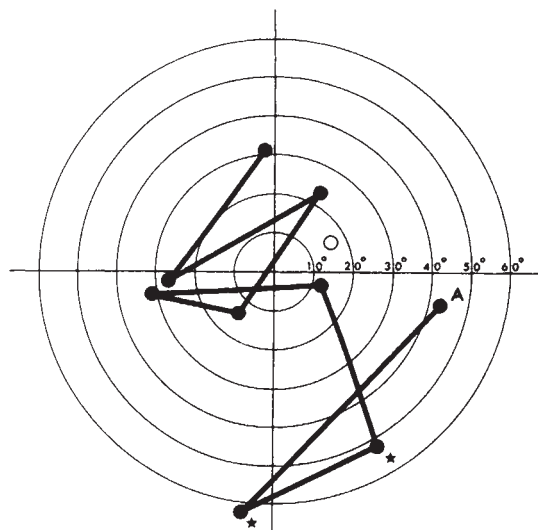


Fig. 1 Location in the visual field of nine receptive fields mapped in a vertical penetration through the right optic nerve beginning at A. The horizontal and vertical lines represent the horizontal and vertical meridians which cross at the area centralis. The open circle marks the projection of the optic disk. The two units marked with stars were recorded simultaneously.

topic order was apparent, although as the electrode descended through the nerve, receptive fields were more likely to be found higher and higher in the visual field. Receptive fields of simultaneously recorded units showed no particular tendency to be aggregated.

These physiological experiments seemed fairly persuasive but the possibility of artefact, due perhaps to uneven slippage or displacement of fibres by the electrode, could not be excluded. Therefore, we determined to corroborate the physiological evidence, by using the recently developed method of tracing anatomical projections by the retrograde transport of the enzyme horseradish peroxidase (HRP), as visualised by tetramethyl benzidine, a highly sensitive chromogen⁷. This technique avoids many of the problems inherent in the older degeneration methods, and it permits a much finer resolution of the fibre arrangement in the optic nerve. Results obtained from one cat will be presented; the findings have been confirmed in two other animals.

An anaesthetised cat was placed in a stereotaxic frame, and a site midway between the dorsal and ventral borders of lamina A of the right lateral geniculate body was located by recording with a tungsten electrode while stimulating with small spots of light in the visual field. The electrode was then replaced with a glass micropipette filled with a 20% solution of HRP in Tris-KCl buffer, the site was verified by recording again, and HRP was passed iontophoretically for 100 min to achieve a localised injection⁸. The animal was allowed to recover and after 44 h was re-anaesthetised and perfused with saline followed by 2.5% glutaraldehyde, 0.5% paraformaldehyde in 0.1 M phosphate buffer solution. A block containing the anterior half of the brain and the optic nerves was cut in serial coronal 100- μ m frozen sections; the retinae were prepared for whole mounts. Tissue was reacted with tetramethyl benzidine and H_2O_2 for HRP activity and examined in the light microscope.

The injection site was centred in lamina A of the right lateral geniculate body, although the surrounding halo of the blue reaction product extended in every direction for about 1.5 mm. The zone of effective HRP uptake was restricted to the core of the injection site, judging from the pattern of cell labelling in the retinae. No HRP activity was present in the right retina; in the left retina (Fig. 2) a small patch of retinal ganglion cells containing HRP was observed. This region, which cor-

responded exactly to the injection site mapped physiologically in the lateral geniculate, occupied about 3.4 mm² or less than 1% of the entire retinal surface area. Granules of HRP product were detected in a total of 3,750 partially filled cells. In the heart of this labelled region eight virtually adjacent β cells were very heavily stained with HRP. Their cell bodies were completely filled and the dendritic tree could be followed out to the tertiary branches in the inner plexiform layer. From each of these eight cells an HRP-filled axon was visible streaming across the retina in a fairly direct course to the optic disk. The eight axons formed a loose bundle, occasionally crossing and intermingling; this made it difficult to be sure of the identity of each axon once it reached the optic disk. No axons could be followed more than 100 μ m from the other more faintly labelled cell bodies.

These same eight axons were readily seen in the very first optic nerve section, just posterior to the optic disk, and followed section by section towards the chiasm. No other axons in the nerve, chiasm, or tract were labelled until a point in the optic tract a few millimetres before the injection site, except for a faint ninth fibre seen briefly for a short segment of the nerve. It proved possible by sketching every few sections to trace the course of each individual fibre from the optic disk through almost the full length of the nerve. Unfortunately, some of the fibres filled erratically for 1.1 mm just before the chiasm and their identities became confused. After the chiasm the eight fibres were assigned new labels and followed in the optic tract up to the lateral geniculate where they became lost in the injection site.

The arrangement of the eight fibres at several points in the optic pathway is shown in Fig. 3. Entering the nerve at the optic disk, the fibres were aggregated, although, relatively speaking, they were farther apart than one would expect from the close proximity of their cell bodies in the retina. For a short distance in the nerve they remained grouped, but with each consecutive section they quickly became more and more widely dispersed, until at 3 mm from the optic disk they were no longer confined to a single quadrant of the nerve. For the remaining 9 mm to the chiasm the fibres maintained a scattered disposition, with less rapid shifts in relative position. In the tract they were also dispersed (not illustrated), with some suggestion of reaggregation as they approached the lateral geniculate body.

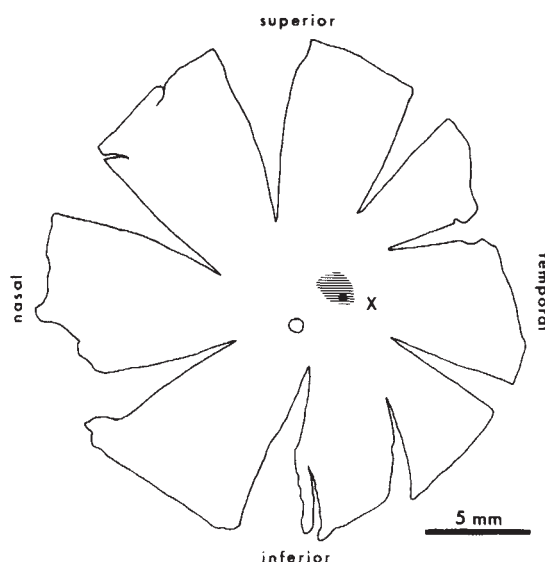


Fig. 2 Outline of a whole mount of the left retina. Cross-hatching indicates the region containing HRP-labelled cells; the black dot marks the location of the eight heavily filled cells. The open circle is the optic disk; the cross represents the position of the area centralis.

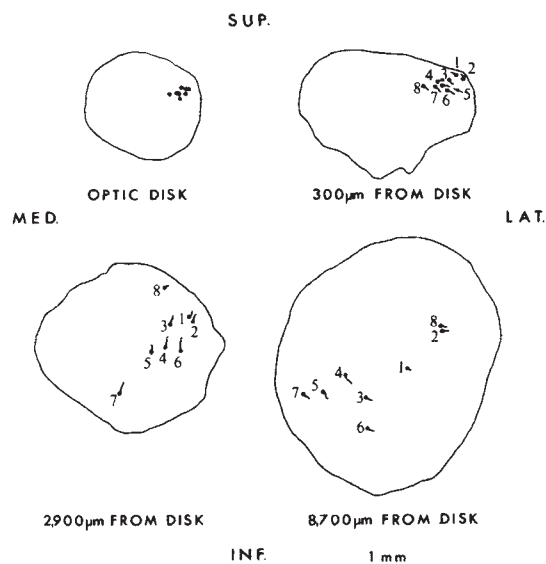


Fig. 3 Position of the eight HRP-labelled fibres at four stages in the optic pathway; their relative thickness has been exaggerated to make them visible. The optic chiasm begins 12,000 μ m from the optic disk.

It is apparent that beyond the first few millimetres no strict retinotopy is present in the cat optic nerve although some crude topography may be preserved; axons of adjacent retinal ganglion cells do not generally travel side by side in the nerve. It might be argued that fibres from these adjacent cells are merely being reassorted in the nerve according to cell type, but we think this is unlikely, especially as in this particular experiment all eight labelled cells happened to be of the same cell type. The fibre pattern in the optic nerve is clearly not based on a system of simple retinal coordinates, although we do not conclude that the fibres are arranged haphazardly. They may be organised in some complicated fashion which is not yet clear. It is interesting, in this regard, that once the fibres diverge quickly in the initial portion of the nerve, they tend to maintain a more constant disposition, hinting that the scatter is somehow controlled.

In the light of our findings, it seems unlikely that the physical arrangement of fibres in the optic nerve and tract could alone account for the retinotopy present in the lateral geniculate body. Conceivably, however, retinotopic order is present as fibres first reach their targets, and their relative positions then shift during subsequent development. The critical information is lacking, namely, the temporal and spatial order of the nerve fibres on arrival at the lateral geniculate body in the embryo. It would further be interesting to know how the fibres of the optic nerve are organised and parcelled into their connective tissue fascicles. Finally, it is perplexing that fibres in the mammalian optic nerve should be scattered, in view of the evidence favouring retinotopic order in the optic nerves of fish and amphibia. One wonders if the non-retinotopic arrangement of fibres in the mammalian optic nerve may be of functional significance.

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Information processing of visual stimuli in an 'extinguished' field

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Lesions of the right parieto-occipital cortex in man produce a variety of behavioural disturbances which interfere with the detection of, and orientation to external stimuli¹. One striking example is extinction to double simultaneous stimulation (DSS), in which presentation of a single stimulus in any area of the visual field results in its accurate description, but lateralised simultaneous presentation of two stimuli, one in each field, results in the verbal description of only the stimulus in the right visual field (RVF)². While extinction to DSS can also be demonstrated in other (non-visual) sensory modalities³, and is occasionally seen following left-hemisphere lesions⁴ our concern here is with visual extinction to DSS following right parieto-occipital lesions whose precise nature is poorly understood although variety of theories have been proposed to account for it⁵⁻⁷. One of the critical, yet unexplored questions about extinction concerns the fate of the extinguished stimulus. The following observations demonstrate that although the extinguished stimulus often goes completely unnoticed by the patient, the patients are able to utilise the extinguished stimulus in an interfield comparison task. Accurate same/different judgments between the visual fields could be made in situations in which the patients did not know the identity of, and at times even denied the presence of, the left field stimulus.

The subjects were four right-handed female patients ranging in age from 56 to 70 years who were selected for study by routine neurologic examination. Although all patients had full visual field capacity when tested by a standard perimetry mapping with a single 1-cm, white object, each patient extinguished LVF stimuli on DSS, and left-sided touch stimuli under double simultaneous tactile stimulation. Each patient was alert and oriented and without language disturbance. Patients were tested on the third hospital day, before therapeutic manoeuvres (two cases). Patient no. 3 received steroids over the 36 hours before testing. Patient no. 4 had a persistent defect that remained unchanged during the weeks following surgery. Angiographic analysis and/or computerised tomography⁸ revealed that all four patients had tumours in the right parietal lobe. (Cerebral angiography is a standard neuroradiologic procedure in which contrast material is injected into each discrete arterial system of the brain. This procedure aids the neurologist in diagnosis and the neurosurgeon in treatment. Computerised tomography is a brain imaging technique.) Neuropathologic analysis of tissue biopsies confirmed the clinical diagnosis of tumour in all patients.

Table 1 Single visual field naming

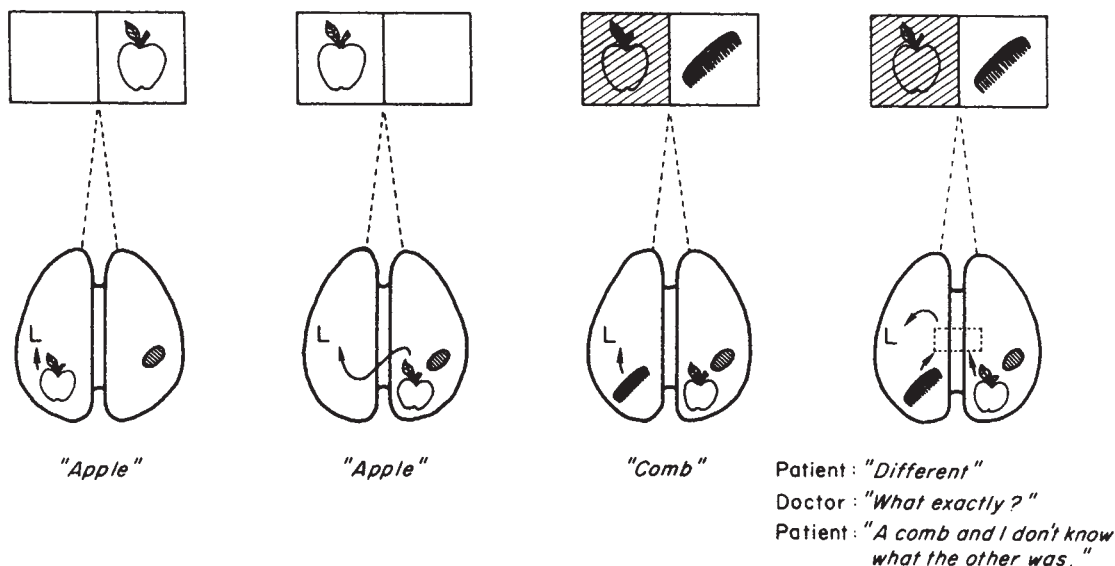
Patient no.	Visual field	
	Left	Right
1	1.00 (15)	1.00 (12)
2	0.94 (16)	0.89 (9)
3	0.86 (14)	1.00 (15)
4	0.91 (33)	0.88 (33)

The proportion of trials that were correctly named by each patient in each visual field is shown. Numbers in parentheses represent total number of trials presented to each visual field. Performance differences between the visual fields were not significant ($t(3) = 1.12$, $P < 0.4$). Variation in total number of trials presented to each patient is described in the text.

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Fig. 1 This composite picture represents each of the experimental paradigms. The two pictures on the left describe the typical left and right single visual field naming trials. The two pictures on the right describe a typical response during the simultaneous bilateral visual field trials, in the 'same/different' paradigm.



In the test conditions, all tasks involved the lateralised presentation of visual stimuli in a manner previously reported⁹. All experiments were carried out in a room in which it was not possible to control the level of background lighting completely. The room was generally as dim as possible, but allowed the experimenter to record the results. The subject was seated 1 m from an opaque screen and instructed to fixate on a dot in the centre of the screen. By means of a standard slide projector fitted with an electronic shutter, stimuli were presented 3° to the right and/or left of fixation for 150 ms. The stimuli were positive photographic slides of three- or four-letter words or simple line-drawn pictures that were rear-projected onto a screen which the subject faced. A representative series of word stimuli subtended 2° of horizontal visual angle and 5° of vertical visual angle. Because of the line-drawn nature of the picture and word stimuli, measurements of luminosity varied less than 0.5% on all bilateral simultaneously projected trials irrespective of similarity or difference. Due to restraints regarding the medical care of these patients, testing sessions were 30 min in duration, and the variability in the number of trials among the patients reflects this primary factor.

Each patient was seated in front of the screen and after fixation on the centre point, the words or pictures were presented randomly to either the left or right visual field. All patients named the stimuli with high accuracy when singly presented to either visual field (see Table 1).

The next series of tachistoscopic test trials was preceded by a group of illustrative trials where words or pictures were, simultaneously, bilaterally projected to the right and left of the fixation point. The patients were shown several such examples under prolonged exposure conditions (5–10 s) so they could identify both stimuli. They were then told that on each subsequent trial there would be two stimuli, one on each side of fixation, and that their task was simply to judge whether the two stimuli were the 'same' or 'different'. In addition to representing quite different items on the 'different' trials, the simultaneously flashed picture stimuli bore no semantic relationships to one another (for example, bicycle and comb). The words flashed on the 'different' trials were three or four letters in length, semantically unrelated, and spelled differently, with, at most, only a single similar letter (HOT and WON for example). On these bilateral DSS trials all patients performed both the same and different comparison with high accuracy (see Table 2).

After verbally reporting in the same/different test situation, the patients were then asked to respond again and name the stimuli. On the trials in which the stimuli were identical, naming of the item in the LVF was an easily accomplished deduction from naming the RVF stimulus. On the same response trials that were correct, there were no LVF naming errors. On the trials in which the stimuli were different, the LVF stimulus could not be

deduced from the RVF stimulus. Patients nos. 1 and 2 could not name the left visual field stimuli, although they had performed accurately on the same/different judgments. In fact, during bilateral simultaneous projection, these two patients could not name any of the left visual field stimuli (Table 3), and asserted further that the task was 'silly', since there was no stimulus in the LVF with which to compare the RVF stimulus. Patients nos. 3 and 4 performed as accurately as the previous two on the same/different judgments, but were able to name some of the left visual field stimuli on the critical different trials (Table 3). Although these latter two patients were more often aware of the presence and nature of the LVF stimuli than the first two patients, all patients' accuracy on 'same/different' judgments was statistically greater than their accuracy at naming the LVF stimulus ($t(3) = 3.53$, $P < 0.05$).

These experimental observations focused on the residual cognitive abilities of four patients with right parietal lesions. Manipulation of exposure duration below 250 ms, the use of single digits, and the use of nonsense pictures and nonsense syllables did not alter the pattern of results. The patients uniformly made accurate judgments when comparing information simultaneously presented to both visual fields, in spite of their inability to verbally characterise with a similar level of accuracy the left visual field information. These data bear relation to the results of studies showing that under certain test conditions normal subjects can be influenced by information they frequently are unable to identify^{10–13}.

Our patients responded to information presented to the extinction field in two ways. In some instances, especially with patients 3 and 4, they felt that something had appeared in the LVF, but they were unable to characterise it. In other instances, as with patients 1 and 2, they were completely unaware that anything had been presented in the LVF. Yet, it is clear that some features of the extinguished stimuli were attended to and

Table 2 Same/different judgment with double simultaneous visual field presentation

Patient no.	Same/different judgments	'Different' trials correct	'Same' trials correct
1	1.00 (17)	1.00 (7)	1.00 (10)
2	0.88 (26)	0.88 (16)	0.90 (10)
3	0.95 (39)	0.96 (25)	0.93 (14)
4	0.90 (68)	0.89 (35)	0.91 (33)

Proportion of same/different judgments that were correct is shown. Correct proportions for trials judged as 'same' and 'different' are separately indicated. Numbers in parentheses represent total number of trials. Each patient compared the bilaterally projected stimuli with high accuracy, and errors occurred with equal frequency on the same and different trials.

Table 3 LVF naming after same/different judgments on double simultaneous visual field presentation trials

Patient no.	Same/different judgments	LVF naming in 'different' trials
1	1.00 (17)	0.00 (7)
2	0.88 (26)	0.00 (16)
3	0.95 (39)	0.48 (25)
4	0.90 (68)	0.23 (35)

The proportion of correct same/different judgments and proportion of different trials in which LVF stimulus was correctly named are shown. Numbers in parentheses represent total number of trials. The accuracy of the same/different judgments was significantly greater than the accuracy of LVF naming ($t(3) = 3.53$, $P < 0.05$).

perceived, for the 'same/different' judgments were accurately made on the basis of these stimuli. It thus becomes difficult to assert that the so-called extinguished stimulus is extinguished at all. Rather, this disturbance seems to involve a selective breakdown in a mechanism through which information which is attended to and perceived reaches some level of neuronal processing which allows for verbal description, if not conscious awareness (see Fig. 1).

Parietal lobe extinction thus offers the possibility of observing, under appropriate test conditions, a breakdown in the flow of information between conscious and non-conscious mental systems. The stimulus comparison task in our study appears to have been carried out at a post-perceptual, pre-verbal level, with only the resultant comparison entering consciousness. Extension of this paradigm may provide a mechanism for studying non-conscious information processing in man.

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A central role for denervated tissues in causing nerve sprouting

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One of the oldest known forms of neuronal plasticity is the ability of peripheral nerves to grow and form functional connections after damage to neighbouring axons¹. Yet the source of the signal which elicits this 'sprouting' remains unknown. In mammalian muscles, paralysis—which gives rise to many of the changes which occur in denervated muscles^{2,3}—causes motor nerve terminals to sprout⁴⁻⁶. Could the inactive muscle fibres (rather than nerve degeneration products, another likely source⁷⁻⁹) be responsible for some of the sprouting found in partial denervation? We confirm in this paper that direct stimulation of a partially denervated muscle inhibits sprouting^{10,11} and show that stimulation does so by activating the denervated fibres. Consequently after partial denervation the same signal as that which causes terminal sprouting in a paralysed muscle is able to spread from the denervated muscle fibres to the nerves on the innervated fibres and initiate terminal sprouting.

The soleus muscles in young adult mice anaesthetised with chloral hydrate were partially denervated by cutting one or both L₄ rami, or were completely denervated by crushing the soleus nerve close to the muscle. Teflon-coated multi-stranded stainless steel wires were implanted on both sides of the soleus muscle for muscle stimulation, or around the sciatic nerve in the thigh for nerve stimulation, drawn under the skin along the tail and brought to the surface. Only one leg per animal was stimulated.

So that the mouse would not feel pain, it was either spinalised at a low thoracic level or the dorsal roots supplying the stimulated leg were cut. The stimulation pattern was 100- μ s square wave pulses of alternating polarity, at 100 Hz for 0.5 s/30 s, or 150 Hz for 0.5 s/10 s (there being no difference in the results obtained). For nerve stimulation the current was set at three times the threshold for ankle extension, delivering 5-20 mA. For direct stimulation currents of 30-40 mA were used. Stimulation was started immediately after the operation. This pattern of stimulation can maintain the acetylcholine sensitivity of a denervated muscle at normal values³.

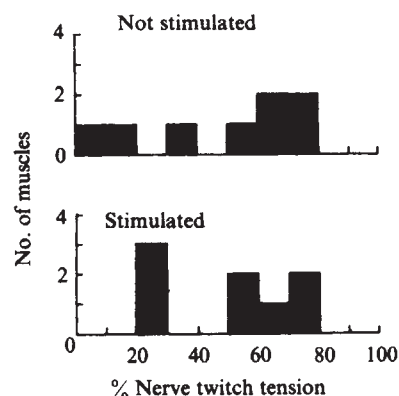


Fig. 1 Histograms of the proportion of the direct twitch tension produced by stimulating the muscle nerve. In eight mice the soleus nerve was crushed close to the muscle on both sides and wires implanted around the left or right muscle. Six to eleven days later both soleus muscles were isolated with their nerves, and the nerve and directly evoked maximal isometric tensions measured. (The crush caused complete absence of nerve-evoked tension in two mice at 2 days.) Two mice were examined at 6 days, five at 7 days and one at 11 days after the nerves were crushed. Clearly, directly stimulating the muscle does not inhibit reinnervation by the crushed nerves. Mean % of direct twitch tension produced by stimulating the nerve = 48% on both sides. This confirms other work^{15,16}, and demonstrates that direct electrical stimulation of a muscle does not inhibit all nerve growth by some mechanism such as damaging the growing tips of axons.

Dorsal root section central to the dorsal root ganglion does not affect the amounts of sprouting in otherwise normally innervated muscles⁹, but spinalisation seems to cause a small increase, presumably due to the muscle inactivity caused by this procedure (unpublished observations).

The soleus muscle and nerve were isolated 6-11 days later and the isometric tensions produced by nerve and muscle stimulation compared. The muscles were then stained in zinc iodide and osmium tetroxide^{5,12}, a procedure which clearly demonstrates both the myelinated and unmyelinated portions of the nerves. Two types of motor axon sprouting have been described, nodal (unmyelinated outgrowths from nodes of Ranvier), and terminal (similar outgrowths from the unmyelinated nerve terminal itself)⁷. The soleus muscle rarely produces much nodal sprouting¹³. Attempts have been made to subdivide terminal sprouting into two forms pre-terminal and ultra-terminal¹⁴ but we have classified the two together. Histological examination of the muscles for terminal sprouting was conducted 'blind', at least 50 endplates per muscle being examined. An endplate was classified as sprouting if the normal smooth contour was broken up by fine outgrowths⁵.

Figure 1 shows the absence of any effect of direct muscle stimulation on the reinnervation of the soleus muscle by the crushed nerves. The nerve produces the same proportion of the direct twitch tension on average, on the stimulated and non-stimulated sides ($48 \pm 8\%$ versus $48 \pm 10\%$; means $\pm$ s.e.m.; see legend for details). This confirms other work^{15,16} and demonstrates that the fairly large currents used do not inhibit nerve growth directly, for example by damaging the growing tips of the axons.

Figure 2 shows the dramatic reduction in terminal sprouting produced by directly stimulating partially denervated soleus muscles. The proportion of nerve terminals with sprouts is reduced from $71 \pm 3.4\%$, $n = 45$, to $11 \pm 1.6\%$, $n = 11$ [$P < 0.00003$, Mann-Whitney U -test].

Figure 2 also shows that partially denervated spinal animals seem to produce less terminal sprouting than partially denervated but otherwise normal animals even when not stimulated. This has been described before¹¹ and the reason is unknown; perhaps the animals were just in a poorer condition overall. Nevertheless there is still a striking difference in the proportion of endplates with sprouts between the stimulated and non-stimulated partially denervated spinal preparations ($11 \pm 1.6\%$ versus $62 \pm 6.8\%$, $n = 13$, $P < 0.001$). Clearly activation of the whole muscle inhibits terminal sprouting very effectively.

One explanation of this inhibition of sprouting is that the denervated muscle fibres remain normal when kept active^{2,3} and that they therefore do not liberate a sprouting agent. However, there is an alternative possibility. The properties of innervated muscle fibres are changed by nerve degeneration products^{3,9,17,18}, and these altered innervated muscle fibres might act as a local stimulus to sprouting. This alteration of innervated muscle fibre properties can probably be prevented by stimulating them in a pattern characteristic of fast muscles³, so our direct stimulation might have been stopping sprouting by preventing the appearance of a local sprouting signal from the innervated fibres.

If this latter hypothesis were correct then stimulation of the nerve—and hence only the innervated muscle fibres in a partially denervated muscle—should also inhibit sprouting. In fact, Fig. 3 shows that stimulating the sciatic nerve on one side in the

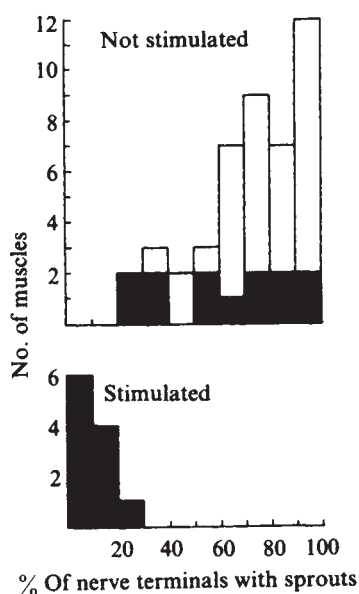


Fig. 2 Histograms of the proportion of nerve terminals with sprouts after partial denervation; above, unstimulated; and below, with direct stimulation of the soleus muscle. Solid bars are data from spinal preparations; open bars, animals normal except for partial denervation. The reduction in terminal sprouting produced by stimulating the muscle directly is dramatic. Mean % of nerve terminals with sprouts falls from $71 \pm 3.4\%$, $n = 45$ to $11 \pm 1.6\%$, $n = 11$, $P < 0.00003$ Mann-Whitney U -test (mean $\pm$ s.e.m. throughout).

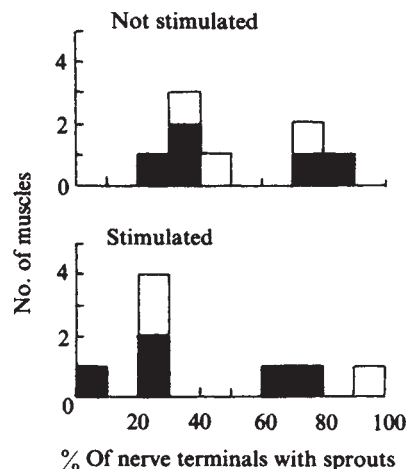


Fig. 3 Histograms of the proportion of nerve terminals with sprouts after partial denervation of both soleus muscles in eight mice. Above, unstimulated; and below, stimulated via the sciatic nerve. Solid bars are data from spinal preparations, open bars are data from mice in which the dorsal roots supplying the stimulated nerve has been cut. Stimulating the nerve to soleus does not seem to influence the amount of sprouting (mean % of terminals with sprouts in control muscles = $50 \pm 9\%$ and in nerve stimulated muscles = $42 \pm 11\%$, and these figures are not significantly different). An important criticism of this experiment is that we have not shown that we were rendering the innervated muscle fibres resistant to the effects of nerve degeneration. However, the muscles were clearly contracting in response to the nerve stimuli. As there is no reason to suppose that the effects of muscle activation via its nerve are different to those of activation via direct electrical current, it can be argued that direct electrical stimulation is not inhibiting terminal sprouting either by activating the nerve or the innervated muscle fibres, irrespective of whether the innervated muscle fibres are made resistant to the effects of nerve degeneration. Another criticism is that the implantation of electrodes around the sciatic nerve might have caused an excess of nerve degeneration on the stimulated side and hence an increase in the amount of sprouting which might mask a reduction due to the stimulation. In fact, the amount of terminal sprouting in a partially denervated mouse muscle is independent of the amount of denervation²².

thigh does not seem to affect the amount of sprouting in a partially denervated muscle, when compared with the contralateral unstimulated muscle ($42 \pm 11\%$ of terminals on the stimulated side showing sprouts, and $50 \pm 9\%$ on the control side).

Clearly this experiment relies on nerve stimulation activating the innervated muscle fibres. In that the muscles were contracting in response to the nerve stimuli this was probably the case, but these results must be interpreted with caution (see Fig. 3 legend).

We have shown, therefore, that direct muscle stimulation inhibits the expected terminal sprouting in a partially denervated muscle, and that this is not due to some damaging effect of electrical current since the same pattern of stimulation does not inhibit reinnervation by a completely crushed nerve. Furthermore, the same pattern of activity in the innervated muscle fibres alone does not seem to inhibit sprouting. Thus, it is activation of the denervated muscle fibres that inhibits the expected terminal sprouting, and this implies that denervated muscle fibres liberate a sprouting signal that spreads through the muscle, for only in this way could nerve terminals on innervated fibres be reached. An alternative explanation that cannot be ruled out would be that normal tissues liberate an antisprouting agent and that denervated tissues no longer produce it. It is also possible that the mere contact of a more proximal part of an intact motor nerve with a denervated muscle fibre might be the requisite sprouting stimulus. In this case the effect would have to traverse the Schwann cell sheath.

We have obtained limited further support for this hypothesis by attempting to show that the signal for terminal sprouting

remains within a partially denervated muscle after nerve degeneration products should have dispersed and the transient rise in acetylcholine sensitivity associated with them should have largely disappeared.

We prevented terminal sprouting for 6 days (the probable duration of the inflammatory response in mice^{19,20}) by directly stimulating the muscle in animals anaesthetised with chloral hydrate for up to 10 h per day whilst stimulation was in progress. (Inactivity due to spinalisation would of itself have caused sprouting after the cessation of stimulation so spinalised preparations could not be used.) Due to the extremely high mortality rate with long-term daily anaesthesia we have only three control animals in which the amount of terminal sprouting was 0, 12 and 28% immediately after the 6 days of stimulation, and two experimental animals with 34 and 60% terminal sprouts 5 and 8 days after stimulation ceased. Thus in these two mice the sprouting signal was probably still present at a time when nerve degeneration effects should be largely over (or certainly much reduced) and the denervated muscle fibres remained as the only major potential source of a stimulus.

Although the present results do not show that denervated tissue is the only source of a sprouting stimulus (and there is evidence to the contrary^{7,8,9,11}) they do suggest that it has an important role, as was proposed many years ago by

Van Harreveld²¹. They also make it plain that in muscle the signal is detectable at a distance by axons on innervated fibres, a fact that was not expected from our study of sprouting in partially blocked muscles²². The maximum distance over which the signal is detectable remains to be determined.

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Competition between foreign and original nerves in adult mammalian skeletal muscle

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It is well established that a normally innervated, uninjured mammalian skeletal muscle does not accept further innervation from a foreign nerve transplanted on to the muscle's extrasynaptic region^{1–3}. Because synapse formation under these circumstances would require the induction of new postsynaptic specialisations, it is not clear whether foreign axons could make connections if they were allowed instead to compete for existing synaptic sites. The capacity of individual endplates to accept multiple inputs has in fact been demonstrated during both normal development^{4,5} and reinnervation of adult mammalian muscle^{6,7}, that is, in conditions involving interactions among recently formed synapses. We report here that even in adult muscles whose normal innervation remains fully intact, a similar susceptibility to further innervation can be expressed following appropriate placement of a transplanted foreign nerve. Moreover, the establishment of foreign synapses can lead to suppression of original inputs.

In aseptic conditions a small motor branch of the superficial peroneal nerve was severed in 75–150-g Sprague–Dawley rats anaesthetised with Nembutal. The proximal nerve stump was then transplanted directly over the endplate region midway along the soleus muscle. Care was taken not to injure the original (soleus) nerve or the muscle surface. After survival times of 1–14 weeks the muscle was removed along with foreign and original nerves for *in vitro* intracellular recordings, as described elsewhere⁸. At the end of the physiological experiment muscles were usually stained for acetylcholinesterase⁹ (AChE) and/or nerve fibres and terminals (J. H. Steinbach, personal communication).

In the majority of experiments (61 of 104) the foreign nerve had drifted away from the placement site over the original endplate band; as expected, foreign innervation of soleus muscle fibres did not occur in these cases, with only rare exceptions. In 21 other experiments, the foreign nerve remained in the general

vicinity of the original nerve but was only loosely attached to the muscle; foreign axons failed to grow into direct contact with soleus muscle fibres, and no synapses were formed. In the remaining 22 experiments, foreign axons successfully grew into the muscle in the immediate vicinity of the original endplates. In all 19 cases of this type, that involved survival times greater than 3 weeks, the presence of foreign-innervated soleus muscle fibres was demonstrated either physiologically, by contractions evoked through the foreign nerve (18 muscles), and/or anatomically, by foreign synapses visible in silver-stained preparations (2 muscles). The total number of foreign-innervated soleus muscle fibres was generally quite small, however, ranging from just a few fibres to several per cent (~100 fibres) of the whole muscle. This low degree of takeover was related mainly to the limited extent to which the foreign nerve grew over the endplate region of the muscle. Within the region of overgrowth, the degree of takeover was much higher and sometimes involved a majority of the superficial muscle fibres that we sampled electrophysiologically.

Several results indicate that the foreign synaptic contacts were made directly at the original endplates, and not just in their general vicinity. No contractions on foreign nerve stimulation were seen in soleus fibres whose endplates were outside the region of overgrowth of foreign axons (Fig. 1a). In silver-stained preparations intramuscular branches of foreign axons could be seen ramifying for several millimetres along the mid-portion of the muscle, but they made identifiable synapses only within the soleus endplate zone. In some cases, careful inspection revealed single endplates that were supplied by both foreign and original nerves (Fig. 1b). Ectopic endplates were seen only rarely in cross-innervated muscles stained for AChE, and no examples of dual endplates were found in 32 fibres teased from the regions of cross-innervation in 6 muscles.

Intracellular recordings from single muscle fibres showed that about half of the foreign-innervated fibres retained their inputs from the original nerve (Fig. 2a). In some cases subthreshold endplate potentials (e.p.ps) were evoked by stimulation of one or both nerves, even though no neuromuscular blocking agents were present. When both inputs were subthreshold, the e.p.ps had similar time courses but different amplitudes (Fig. 2b), providing further evidence that foreign and original synapses, were in close proximity. In the remainder of foreign-innervated fibres, however, stimulation of the original nerve evoked no visible response (Fig. 2c). Many of these recordings were sufficiently close to the endplate region for subthreshold

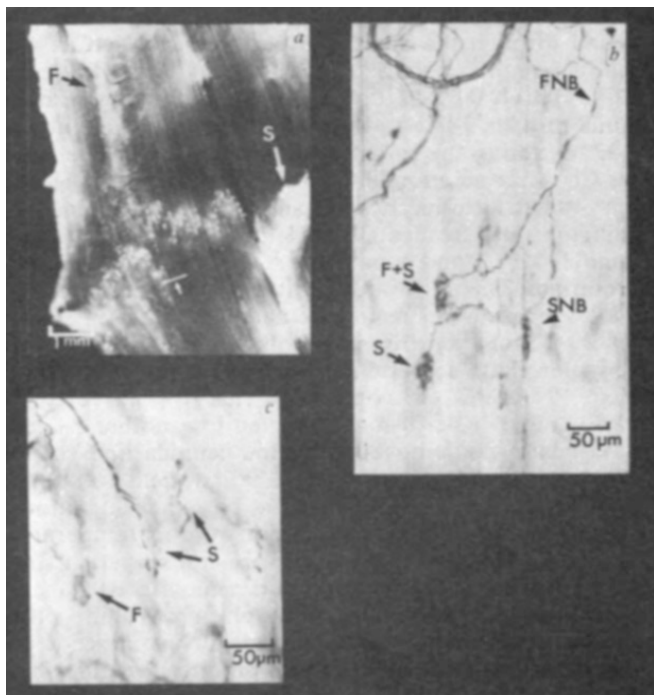


Fig. 1 Morphological evidence for foreign synapses at soleus endplates. *a*, Dark-field photograph of soleus muscle stained for AChE, and showing soleus nerve insertion (S), overgrowth of transplanted foreign nerve (F) and soleus endplate band (white dots). Bar with arrow underneath denotes the band of soleus muscle fibres in which the foreign nerve elicited contractions. This coincides with the region in which foreign axons grew in the immediate vicinity of soleus endplates. Occasionally, in other experiments, ectopic AChE was seen on a few fibres. In six cases, all from two muscles, these were actual ectopic endplates (perhaps resulting from injury to a few fibres¹⁶), but in all other cases the AChE was clearly nonsynaptic and distributed like normal tendon AChE; teasing of single fibres indicated that the operation had induced bifurcations and/or abnormal terminations of fibres^{17,18}. *b*, *c*, Silver-stained preparations from muscles in which the foreign nerve reached the soleus endplates. *b*, A foreign nerve branch (FNB) and an intramuscular branch of the soleus nerve (SNB) both innervate a single endplate (F+S). Also visible is another endplate innervated only by the soleus nerve (S). *c*, Several endplates are visible, two of which (S) are innervated only by the soleus nerve, and one of which (F) is innervated only by the foreign nerve.

responses, if present, to have been seen; their absence indicates that complete suppression of original synapses had occurred. As we found examples in silver-stained preparations of endplates in the vicinity of the original nerve that were innervated only by the foreign nerve (Fig. 1*c*), it is likely that the suppression of original inputs involves the retraction and eventual disappearance of soleus nerve terminals. Figure 2*d* shows the overall incidence of subthreshold relative to suprathreshold responses for the 59 fibres innervated only by the foreign nerve and for the 54 dually innervated fibres.

A comparison of results obtained at early (4–6.5 weeks) and late (7–14 weeks) survival times showed no large differences in the percentages of foreign-innervated fibres which retained inputs from the soleus nerve. Although this might reflect the early establishment of a stable outcome at each endplate, it seems more likely that the interaction between nerves is a dynamic process; thus, endplates which are dually innervated at one stage might later become singly innervated, and endplates taken over completely by the foreign nerve might themselves be subject to a re-establishment of original inputs.

It was important to ascertain whether our surgical procedure led to any transient denervation or traumatization of synapses made by the soleus nerve that might have rendered muscle fibres abnormally susceptible to cross-innervation. As denervated soleus muscle fibres are readily cross-innervated at ectopic

sites³, the absence of such ectopic endplates in almost all muscles argues against the occurrence of any substantial degree of denervation following surgery. Furthermore, in control experiments involving placement of a freshly excised segment of the peroneal nerve over the soleus endplate region, no signs of denervation were seen in intracellular recordings from muscles examined at 1, 2, 3, 8 and 12 d after the operation. The morphology of endplates examined with silver staining 4, 5 and 16 d after such a dead nerve implant was also indistinguishable from normal. Thus, our particular surgical procedure apparently did not result in the markedly shrunken endplates and extensive terminal sprouting reported by others to occur in the vicinity of degenerating tissue¹⁰. Additional evidence against a major role of nerve trauma came from control experiments in which the foreign nerve was crushed in the popliteal fossa either at the time of the transplant or in a separate operation 2 weeks later. Under these circumstances, foreign axons presumably did not reach soleus endplates until well after recovery from any trauma to the original nerve. Nonetheless, successful cross-innervation by the foreign nerve took place in all five experiments (out of 24 attempts) in which the foreign nerve grew into the endplate region of the muscle. No obvious differences were detected between these crushed-nerve transplants and our normal transplants in degree of cross-innervation. Moreover, the soleus nerve inputs were reduced or absent in 11 of 25 cross-innervated fibres sampled from these muscles; this incidence of suppression was not significantly different from that seen in our normal transplants ($P > 0.2$; χ^2 test).

The ability of foreign axons to suppress original nerve synapses may be related to a competitive advantage of terminals belonging to neurones with relatively few peripheral contacts^{5,11,12}. There is little if any advantage of appropriate relative to inappropriate skeletal motoneurons in the reinnervation of adult mammalian muscle¹³. Thus, in the present situation foreign axons deprived of all their peripheral connections can compete successfully with and eventually displace soleus axons innervating their normal complement of muscle

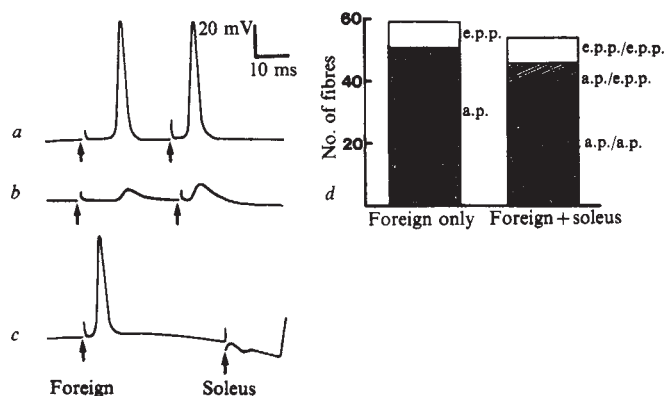


Fig. 2 Physiology of foreign-innervated muscle fibres. *a*, Intracellular recording from a muscle fibre which responded with action potentials (a.p.s) to stimulation of both foreign (first arrow) and soleus (second arrow) nerves. *b*, Recording from a fibre responding with an endplate potential (e.p.p.) to stimulation of either foreign or soleus nerves. *c*, Recording from a fibre which responded with an a.p. to foreign nerve stimulation and gave no response to stimulation of the soleus nerve. The field potential seen a few ms after the second arrow was also recorded when the electrode was outside the muscle fibre. *d*, A bar histogram summarizing the physiological results. On the left are results from fibres innervated only by the foreign nerve, indicating the incidence of e.p.p. (open) and a.p. (stippled) responses to nerve stimulation. On the right are results for the dually innervated fibres, showing the number of fibres giving a.p.s to both nerves (stippled), e.p.p.s to both nerves (open), or an a.p. to one with an e.p.p. to the other (hatched). For the 10 fibres showing mixed a.p./e.p.p. responses, 8 had e.p.p.s to foreign nerve stimulation, and 2 to soleus nerve stimulation.

fibres, and their success might be related to this difference in number of pre-existing connections. In lower vertebrates, the interactions between foreign and original nerves are biased by relative affinities of motor nerves for appropriate muscles¹³, but here, too, the final outcome of any particular competitive situation may be affected by the overall peripheral fields controlled by different nerves.

Previous examples of synapse elimination in the adult nervous system have involved the loss of synapses which themselves were newly formed (after sprouting¹² or regeneration^{6,7}) and thus possibly immature. The present study suggests that mature neuromuscular synapses are also labile in appropriate conditions, possibly even in completely normal muscles¹⁴ (but see ref. 15). If such lability exists for synapses in the central nervous system it could have a significant role in various types of normally and abnormally induced neural reorganisation.

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Contractile force measured in unskinned isolated adult rat heart fibres

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A number of investigators have succeeded in preparing isolated cardiac cells by enzymatic digestion which tolerate external $[Ca^{2+}]$ in the millimolar range^{1–6}. However, a persistent problem with these preparations is that, unlike *in situ* adult ventricular fibres, the isolated fibres usually beat spontaneously. This spontaneity suggests persistent ionic leakage not present *in situ*. A preferable preparation for mechanical and electrical studies would be one which is quiescent but excitable in response to electrical stimulation and which does not undergo contracture with repeated stimulation. We report here a modified method of cardiac fibre isolation and perfusion which leaves the fibre membrane electrically excitable and moderately resistant to mechanical stress so that the attachment of suction micropipettes to the fibre is possible for force measurement and length control. Force generation in single isolated adult rat heart fibres is consistent with *in situ* contractile force. The negative staircase effect (treppe) characteristic of adult rat heart tissue is present with increased frequency of stimulation. Isometric developed tension increases with fibre length as in *in situ* ventricular tissue.

Hearts of anaesthetised adult rats (250–500 g) were excised, cannulated through the aorta and perfused with a Ca^{2+} -free solution of (mmol l⁻¹) NaCl, 130; KCl, 4.0; MgCl₂·H₂O, 1.0; NaHCO₃, 10.0; NaH₂PO₄, 0.435; glucose, 5.56. The perfusate was equilibrated to 98% O₂–2% CO₂ at pH 7.0 and maintained at 30–33 °C during the initial perfusion. Following perfusion with the Ca^{2+} -free solution for approximately 5 min, a digestive medium was introduced containing 0.1% collagenase (Worthington Biochemical), 0.2% hyaluronidase (Sigma), 0.25 mmol l⁻¹ Ca^{2+} , and 4 mmol l⁻¹ ATP added to the original saline solutions. This perfusate was titrated with NaOH to give a pH of 7.2. Perfusion was maintained at 3 ml min⁻¹.

After 20–35 min the digestion solution was replaced with Tyrode's phosphate solution containing 1 mmol l⁻¹ Ca^{2+} and 20 mmol l⁻¹ NaH₂PO₄ (without ATP). This gives a free Ca^{2+} of about 140 μmol l⁻¹. Perfusion continued for another 2–3 min. Then the heart was removed from the cannula and cut into segments with scissors. At this stage, cardiac cells were easily dispersed from these chunks of tissue by gentle agitation in oxygenated Tyrode's phosphate solution at room temperature. Aliquots of the dispersed solution were transferred to fresh phosphate solution on a cover slip on the microscope stage.

Electrical excitability was tested by placing two fine silver-silver chloride electrodes on either side of a quiescent cell. Current pulses 1–3 ms in duration produced rapid phasic contractions in excitable cells without inducing spontaneous slowly propagating irregular contractions characteristic of dying or apparently leaky cells.

The only form of attachment which our cells will tolerate at forces of the order of isometric contraction is to draw the ends of the cell into closely fitting micropipettes. The tip of the pipette must be fire polished so that the wall thickens at the tip (Fig. 1a). For attachment the end of a cell was pulled a short distance into the smooth constricting orifice of the micropipette by suction. This compressive force on the cell so far has been the only method of attachment which the cell will tolerate without

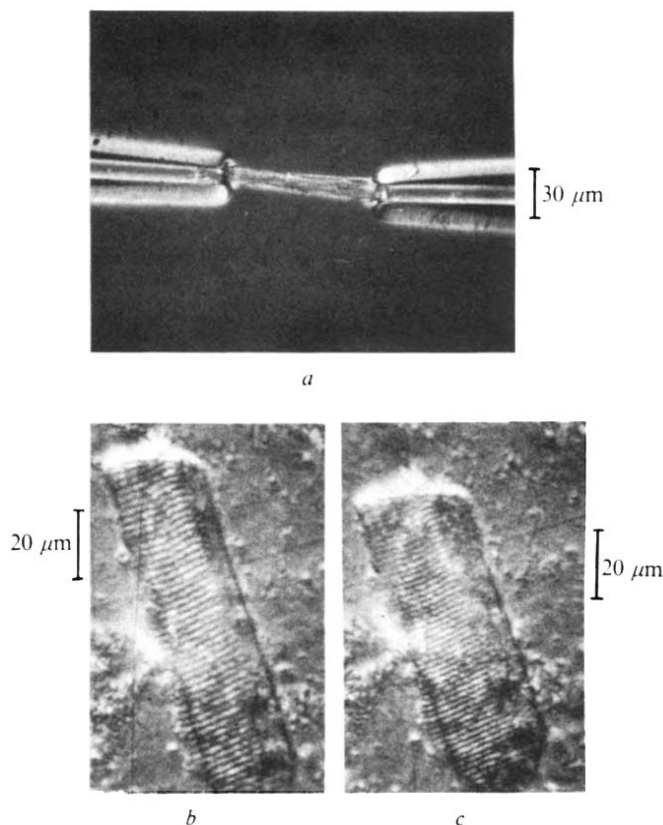


Fig. 1 a, Micrograph of an isolated cardiac fibre held by two suction micropipettes. b, Micrograph of quiescent isolated cardiac fibre. c, Same fibre photographed near the peak of contraction.

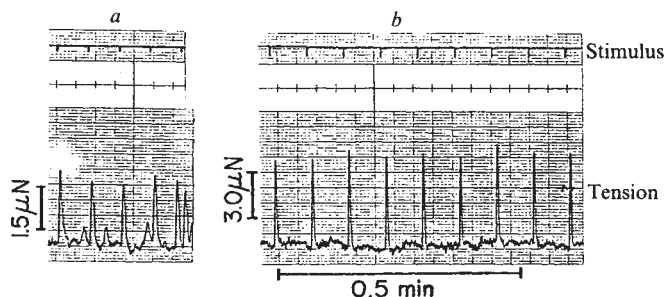


Fig. 2 *a*, Isometric contractions from a single isolated cardiac fibre. Upper trace, stimulus; lower trace, contractile force. Low phosphate solution. *b*, Similar to *a*, except in high phosphate solution. No spontaneous beats are present.

induction of spontaneous activity and subsequent deterioration of the cell.

The necessity to use suction pipettes for attachment introduces a relatively large mass and, therefore, inertial artefact into the transducer system. To obtain an adequate sensitivity for registration of single cell twitch force ($0.1\text{--}10\ \mu\text{N}$) a micropipette was attached to a stylus whose position was detected by a photodiode system. Suction to the transducer micropipette was provided by making the stylus from stainless steel tubing (0.050 inches diameter). Suction for the other pipette was provided by tubing mounted on a micromanipulator. The entire system was mounted on an air suspension table. The r.m.s. noise level (with the signal fed through a 5-Hz filter) was equivalent to $0.15\ \mu\text{N}$.

Fibres in a variety of mechanical and structural states are produced by this method of isolation. Thirty minutes after digestion about 10% of the excised fibres are rod-like but quiescent, another 10% beat spontaneously and the remainder have begun to show some form of contracture. Of the rod-like fibres, tandem doublets or triplets sometimes adhere together sufficiently strongly to bear contractile tension.

Figure 1*b* shows an example of a quiescent unmounted cell in $1\text{ mmol l}^{-1}\text{ Ca}^{2+}$ with uniform striations of $\sim 1.9\ \mu\text{m}$ length. Figure 1*c* shows the same cell in response to a stimulus with the photograph taken near the peak of a twitch. The cell is unloaded. Shortening to about $1.6\ \mu\text{m}$ has taken place. The uniformity of striation spacing is well preserved during the contraction. Rise time of the twitch is about 200 ms at 23°C with relaxation occurring in an additional 200 ms. Relaxation to $1.9\ \mu\text{m}$ occurs rapidly and completely even in the unloaded cell.

Figure 2*a* shows a series of isometric contractions of a cell stimulated at 13.3 min^{-1} . Resting tension is approximately 3 mN mm^{-2} . The contractions in Fig. 2*a* were obtained in $250\ \mu\text{mol l}^{-1}\text{ Ca}^{2+}$ and only buffer phosphate ($0.435\text{ mmol l}^{-1}\text{ H}_2\text{PO}_4$). The preparation was excitable and followed the stimulus but other spontaneous contractions were also present. Figure 2*b* shows the responses with 20 mmol l^{-1} phosphate and 1 mmol l^{-1} total $[\text{Ca}^{2+}]$ (titrated to pH 7.2). Calculated free Ca^{2+} in this solution was $140\ \mu\text{mol l}^{-1}$ but developed force was comparable to that in 1 mM free $[\text{Ca}^{2+}]$. The preparation is now stable, that is, it follows the stimulus without the extra spontaneous contractions and the contractions are larger in amplitude.

These fibres show great potential for a preparation in which contractile force and sarcomere length can be directly monitored.

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Suppression of natural antitumour defence mechanisms by phorbol esters

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Chemical carcinogenesis in certain tissues occurs in at least two stages: initiation, producing irreversible tissue alterations, and promotion (by agents, themselves non-carcinogenic), enhancing the outgrowth of transformed cells^{1,2}. Among the various tumour-promoting compounds isolated from croton oil, phorbol-12-myristate-13-acetate (PMA), is the most potent³. Cell culture studies have shown that the phorbol diesters and structurally related substances induce a variety of dramatic changes in diverse eukaryotic cells. Spreading, differentiation, metabolism, DNA synthesis, expression of cell surface glycopeptides, deoxyglucose transport, as well as polyamine, plasminogen activator and ornithine decarboxylase activity are altered^{4–6}. These findings indicate that tumour promoters potentiate expression of the transformed phenotype in cells already malignantly transformed and also induce reversible manifestations of the transformed phenotype in normal cells⁴. It is not known whether these agents additionally influence host effector systems involved in antitumour resistance^{7–11}. The present study is concerned with the effects of PMA on spontaneous *in vitro* cytotoxicity by macrophages and/or natural killer (NK) cells, and on the resistance to rat fibrosarcoma *in vivo* considered to depend on these normal effectors, in particular on mononuclear phagocytes¹².

Recent evidence suggests that there are at least two major cell populations capable of expressing spontaneous *in vitro* cytotoxicity against tumour targets: mononuclear phagocytes (monocytes, macrophages), a population of adherent, phagocytic, motile cells widely distributed in tissues and capable of

Table 1 Spontaneous *in vitro* cytotoxicity expressed by rat peritoneal cells is suppressed by PMA

Target cell type (and origin)†	Origin of effector cells			
	Adherent peritoneal cells from untreated controls		Adherent peritoneal cells obtained 7 d after <i>C. parvum</i> * inoculation	
	Alone	+PMA (10^{-7} M)	Alone	+PMA (10^{-7} M)
DMBA-12 (DA rat)	21 (± 11)	11 (± 8)	33 (± 10)	19 (± 10)
Py-14 (DA rat)	36 (± 16)	11 (± 8)	45 (± 14)	19 (± 9)
P-815 (DBA/2 mouse)	37 (± 10)	11 (± 8)	43 (± 12)	22 (± 10)

Adherent DA rat peritoneal cells (2×10^6) were interacted in 35-mm plastic Petri dishes for 36 h with 2×10^5 target cells which had been prelabelled with ^{14}C -thymidine (ref. 16). Effects on viability are expressed as net percentage of ^{14}C -thymidine released and values are means ($\pm$ s.d.) of at least 12 determinations, each performed in triplicate. Spontaneous release of ^{14}C -thymidine (8–14% which has been considered) was not increased by PMA.

* 3 mg heat-killed *C. parvum* organisms per rat i.p.

† Target cells were grown and interacted as described¹⁶.

In each case, the presence of PMA significantly ($P < 0.001$; Student's *t*-test) decreased cytotoxicity.

interacting with a variety of target cells of diverse origin¹³⁻¹⁶, and NK cells, nonadherent, nonphagocytic cells found predominantly in spleen and blood and capable of killing a more limited range of targets, including leukaemia and lymphoma cells^{17,18}. Expression of cytotoxicity by these effector cell populations is markedly enhanced by certain microbial pathogens and their products.

In pilot experiments, adherent DA rat peritoneal effector cells ('macrophages') collected from untreated controls or 7 d after intraperitoneal inoculation of *C. parvum* were interacted for 36 h with syngeneic or xenogeneic target cells (Table 1). In these experimental conditions, a lag phase is involved in the consistent expression of natural macrophage cytotoxicity. It was found that cytotoxicity expressed by effectors derived either from normal or from *C. parvum*-treated rats was significantly diminished in the presence of PMA (1×10^{-7} M). In other experiments it was established that the extent to which natural macrophage cytotoxicity was suppressed, was dependent on the actual PMA concentration (Table 2). Even in concentrations as low as 1×10^{-9} M, PMA clearly reduced macrophage-mediated target cell lysis; phorbol had no such effect. As interpretation of such cytotoxicity tests is complicated by the possibility that nucleosides produced by mononuclear phagocytes might interfere with the assay^{6,19}, the basic findings have been ascertained by both the ¹⁴C-thymidine and the ³H-proline 36 h-test²⁰; results obtained with these two cytotoxicity tests corresponded.

To assess whether macrophages or tumour cells were the target of drug action, effector and/or target cells were

Table 2 Effect of concentration of PMA and phorbol on lysis of Py-14 DA rat tumour cells by activated macrophages

	Actual concentration of PMA or phorbol (M)				
	Control	10^{-6}	10^{-7}	10^{-8}	10^{-9}
AM + target	74(±9)				
AM + target + PMA		40(±6)*	48(±7)*	55(±10)*	56(±8)*
AM + target + phorbol		68(±8)	68(±10)	69(±8)	70(±6)

Experimental conditions were as in Table 1. Values representing net percentage of ¹⁴C-thymidine release are means (±s.d.) of at least 10 determinations, each performed in triplicate. Spontaneous release of ¹⁴C-thymidine (7–15% which has been considered) was not increased by PMA, phorbol and/or dimethyl sulphoxide (0.0033% vol/vol).

* These values were significantly different ($P < 0.001$; Student's *t*-test) from controls.

pretreated for various intervals with PMA, washed and then interacted. Pretreatment of DMBA-12 or Py-14 tumour cells with PMA for varying intervals in no case resulted in increased susceptibility of targets to macrophage-mediated lysis; pretreatment with high concentrations of PMA rendered target cells rather less susceptible (Table 3), possibly due to an inhibitory effect of PMA, bound to or released from targets, on effector cells. Neither the proliferation nor the viability of target cells was affected by 10^{-9} – 10^{-7} M PMA (not shown). On the other hand, the capacity of effector cells to express spontaneous cytotoxicity was markedly diminished following pretreatment with PMA for 4 (Table 3) or 18 h. After washing, this reduction in the lytic potential of effector cells was preserved for at least 18 h; after 36 h intermission the results were variable. These data strongly suggest that the PMA effect is primarily on the macrophage and that it decays rather slowly. Recent work attests to the capacity of PMA to induce in macrophages a variety of structural and metabolic perturbations such as vacuolisation, increase in oxygen consumption, oxidation of 1-¹⁴C- and 6-¹⁴C-glucose, release of superoxide, hydrogen peroxide, prostaglandins and/or thromboxane^{8-10,21}, and alterations in the affinity of receptors for epidermal growth factor¹¹.

The present findings that PMA depresses natural cytotoxicity expressed by macrophages would seem at first to be in conflict with recent reports that PMA, in a similar dose range, triggers granulocytes and activated macrophages to lyse various target cells^{8,21}. There are, however, considerable differences between PMA-triggered short-term and spontaneous long-term macrophage-mediated cytotoxicity. PMA-induced short-term cytotoxicity is mediated by hydrogen peroxide and is readily abrogated by catalase²¹, whereas long-term spontaneous tumouricidal activity is unaffected by catalase (not shown). Moreover, the target cells used in the present work were fairly resistant to H_2O_2 ; whereas the particularly susceptible P-388 cells were effectively killed by $5-1 \times 10^{-5}$ M H_2O_2 , at least one log range higher concentration of H_2O_2 is required to lyse the rat tumour cells (not shown). All available evidence thus suggests that hydrogen peroxide released from *C. parvum*-induced activated rat macrophages does not reach the critical concentrations required to kill by PMA the targets used. This may be the reason for our consistent inability to demonstrate PMA-induced short-term lysis in the present *in vitro* model system.

As a measure of NK activity, spleen cells from normal Lewis rats were interacted for 4.5 h with mouse lymphoma-derived YAC-1 cells shown to be highly sensitive to lysis by NK cells²². The target cells used in the previous experiments to monitor macrophage-mediated cytotoxicity (Table 1) were resistant to short-term lysis by spleen cells. Moreover, YAC-1 cells were also considerably more resistant to H_2O_2 than P-388 cells (C. F. Nathan, personal communication) and NK activity was unaffected by catalase (not shown). The results depicted in Fig. 1

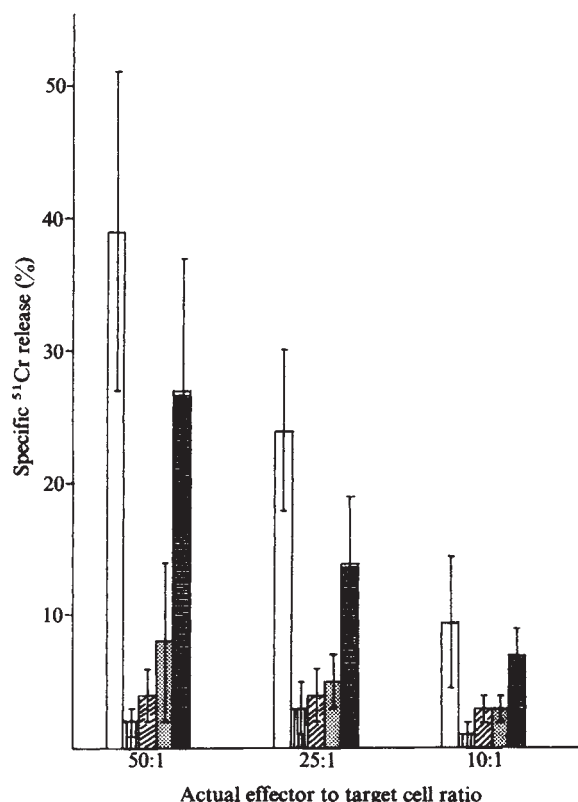


Fig. 1 Suppression of 'natural killer' lysis of YAC-1 targets by PMA. 2×10^4 ⁵¹Cr labelled YAC-1 cells ($50 \times \mu$ Ci per ~ 10 target cells; 40 min at 37°C) were interacted for 4.5 h with 2×10^5 , 5×10^5 and/or 10^6 Lewis rat spleen (NK) cells. Results are expressed as percent specific ⁵¹Cr release²². □ Control (spleen cells + YAC-1 targets); ▨ PMA 10^{-6} M; ▩ PMA 10^{-7} M; ■ PMA 10^{-8} M; ■ PMA 10^{-9} M. Values are means of at least 10 determinations, each performed in triplicate. * These values are statistically significantly different ($P < 0.001$; Student's *t*-test) for controls.

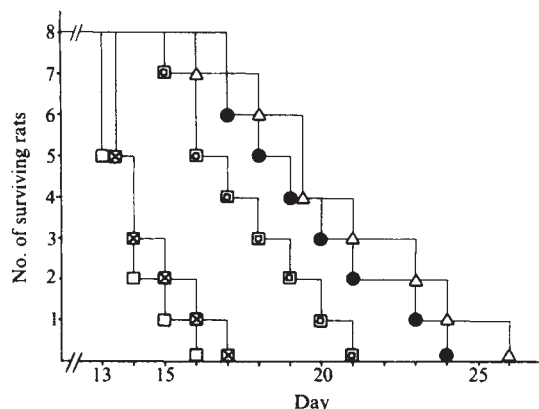


Fig. 2 Promotion of tumour growth by the phorbol ester, PMA. ● Controls (survival 20 ± 3 d); □ PMA $5 \mu\text{g}$ per rat (survival 14 ± 1 d)*; × PMA $2.5 \mu\text{g}$ per rat (survival 14 ± 2 d)*; ○ PMA $1.0 \mu\text{g}$ per rat (survival 18 ± 2 d); △ phorbol $5 \mu\text{g}$ per rat (survival 21 ± 3 d). PMA and phorbol were administered i.p. on day -1. On day 0, 10^3 DMBA-12 tumour cells growing in ascites form²³ were inoculated i.p. * These values are statistically significantly different ($P < 0.001$; Student's *t*-test) from controls.

show that PMA, in concentrations of 1×10^{-8} M and higher, significantly suppressed or abolished expression of cytotoxicity by NK cells at various effector to target cell ratios.

DMBA-12 tumour cells are of low immunogenicity and resistant to the lytic activity of NK cells. Furthermore, anti-macrophage agents, such as silica particles and the high molecular weight sulphated polygalactose carrageenan, effectively enhance tumour growth in this DA rat fibrosarcoma model provided they are administered briefly before or on the day of intraperitoneal tumour cell challenge^{12,23}. These findings were interpreted as indicating that resistance to this particular rat fibrosarcoma is essentially dependent on mononuclear phagocytes. The intraperitoneal (i.p.) inoculation of rats with 10^3 tumour cells growing in ascites form led to progressive tumour growth and death within approximately three weeks (Fig. 2). Local PMA administration on day -1 diminished tumour resistance in a dose-dependent fashion (Fig. 2); on the other hand, phorbol (5×10^{-6} g per rat i.p. on day -1) did not enhance tumour growth (not shown). The consistency of these findings was ascertained in 16 PMA and 7 phorbol experiments. As for other agents affecting host tumour resistance¹², the capacity of PMA to abrogate resistance was markedly dependent on the

interval between its administration and challenge with tumour cells. Enhancement of tumour growth was most pronounced when PMA was administered locally shortly before or at the time of intraperitoneal tumour cell challenge. With increasing interval between tumour cell inoculation and administration of this agent, the tumour-promoting effect diminished or disappeared¹².

These findings show that the tumour-promoting agent PMA suppresses two *in vitro* manifestations of natural antitumour defense mechanisms; that is, the tumouricidal activity of both macrophages and NK cells, probably by selectively acting on effector cells. PMA also abrogates host tumour resistance *in vivo*. It can hardly be fortuitous that an exceedingly active tumour promoting agent also profoundly suppresses the two primary natural antitumour effector systems. The present findings may thus be relevant to understanding the fundamental mechanisms involved in carcinogenesis.

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Table 3 Effect of PMA on expression of spontaneous cytotoxicity by macrophages *in vitro*

Actual concentration of PMA (M)	AM + target + PMA	(Target + PMA 4 h)§ + AM	(AM + PMA 4 h)§ + target
none (control)	47 (±5)	47 (±10)	48 (±5)
10^{-6}	18 (±11)*	24 (±12)*	16 (±10)*
10^{-7}	15 (±9)*	33 (±9)*	17 (±10)*
10^{-8}	19 (±11)*	40 (±9)	33 (±15)*
10^{-9} (±16)*	44 (±11)	40 (±15)	

Py-14 DA rat tumour cells were used as targets. Experimental conditions were as in Table 1. Effector and/or target cells were first incubated for 4 h, washed four times (§), and then interacted for 36 h. Spontaneous release of ^{14}C -thymidine (5-13% which has been considered) was not increased by PMA and/or dimethyl sulphoxide (0.0033%; vol/vol). Values representing net percentage of ^{14}C -thymidine release are means (±s.d.) of at least 12 determinations, each performed in triplicate.

* These values were significantly different ($P < 0.001$; Student's *t*-test) from controls.

Cell-mediated immunity in the liver of mice vaccinated against malaria

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Mice can be protected against several species of lethal malaria infection by vaccination¹, and their recovery correlates well with increased anti-malarial antibody levels, particularly IgG (ref. 2). However, there is also a good correlation between protection by vaccines and priming for delayed-type hypersensitivity in the skin³⁻⁵, although there is no obvious explanation for this effect. We now report an apparent relationship between protection and a cell-mediated immune response involving the migration of various types of cell capable of killing malaria parasites *in vitro* to the liver. We suggest that the effect of vaccination is to bring together parasites, specific antibody and nonspecific cytotoxic cells, and that the liver may be a major site for their interaction.

Table 1 Passive transfer of increased liver accumulation pattern by cells (but not serum) from vaccinated mice

Mice	Passive transfer	Bone marrow		Lymph node	
		Injected cells in liver after 24 h (%)	Increase above uninfected controls (%)	Injected cells in liver after 24 h (%)	Increase above uninfected controls (%)
Controls uninfected	—	24.47 ± 0.6		33.47 ± 0.64	
Day 7 infected	—	37.63 ± 1.2	+13.16	36.15 ± 1.24	+2.68
Vaccinated-day 7 infected	—	*44.1 ± 2.1	+19.63	*44.1 ± 2.6	+10.63
Day 7 infected	Vaccinated cells	*44.76 ± 1.8	+20.29	*42.42 ± 2.2	+8.95
Day 7 infected	Vaccinated-recovered serum	39.94 ± 0.9	+15.47	34.27 ± 1.7	+0.8

The accumulation of normal labelled bone marrow and lymph node cells in the liver was measured 7 days after infection with 10^4 *P. yoelii* parasites (for details see Fig. 2a). For passive transfer, 80×10^6 mixed spleen and lymph node cells from vaccinated uninfected donors were injected i.v. into normal mice 1 week before infection; alternatively 1.5 ml of high-titre antiserum from vaccinated-infected-recovered mice were injected on day 6 of infection. The values shown are the mean ± s.e. of groups of 10–45 mice.

* Significantly different from the value for infected mice ($P < 0.02$).

We have used (BALB/c × C57Bl)F₁ mice and two lethal malaria parasites—a lethal variant of *Plasmodium yoelii* (strain 17X) and *Plasmodium berghei* (ANKA strain)⁶. Vaccination consisted of a single intravenous (i.v.) injection of 10^8 parasitised red cells lysed with saponin and fixed with formalin combined with 10^8 *Bordetella pertussis* organisms¹. A vaccine made from *P. yoelii* protected all mice against death from a challenge infection with 10^4 live *P. yoelii* parasites; parasitaemia developed normally for about 5 days, after which damaged parasites (crisis forms) were seen and parasites disappeared from the blood by day 7, leaving the mice apparently parasite-free and immune to reinfection. In the case of *P. berghei*, protection by vaccination was less complete: about 60% of the mice recovered 2–3 weeks after challenge and the remainder died¹. Splenectomy after vaccination has been found to delay but not prevent recovery from *P. yoelii*. Passive transfer of sufficient high-titre serum (1.5 ml) taken from *P. yoelii* vac-

inated-challenged-recovered mice to confer on normal mice a similar antibody titre, helped them to eliminate a subsequent infection, but only 2–3 weeks after challenge; delaying the injection of serum did not alter this pattern of recovery⁷. The injection of 'recovered' spleen cells from the same donors together with the serum improved survival but did not accelerate the clearing of parasitaemia, which still took an average of 2 weeks (Fig. 1). This suggests that changes in the infected host, other than antibody, are needed before parasites can be eliminated.

During the study of delayed hypersensitivity, we noticed that injected bone marrow cells accumulated in the spleen and liver of *P. yoelii*-infected mice³. On further investigation we have found that both labelled bone marrow and lymph node cells accumulated in increased numbers in the spleen a few days after infection, but that later there was a larger and more prolonged increase in accumulation in the liver and a reduction of accumulation in the spleen to below control levels (Fig. 2). In *P. yoelii* infection (Fig. 2a) these changes were further modified by previous vaccination; in particular the accumulation of bone marrow cells in the liver reached a peak (day 7) earlier than in unvaccinated mice (day 10). Lymph node cells showed roughly the same pattern. With *P. berghei* (Fig. 2b) the general level of increase was lower, and vaccination had less effect, apart from a brief increase in bone marrow accumulation at day 5. There was thus a good correlation between the degree of accumulation in the liver and that of delayed hypersensitivity in the skin which was stronger in *P. yoelii* than in *P. berghei* vaccinated mice³. There was also a close correlation between the timing of accumulation in the liver and of recovery from infection—7 days in *P. yoelii*-vaccinated mice, 10 days or more in unvaccinated serum-transferred mice (Fig. 1), and over 2 weeks (if at all) in *P. berghei*-vaccinated mice. The pattern of accelerated cell accumulation in the liver characteristic of *P. yoelii*-vaccinated mice could be transferred to unvaccinated mice by lymphoid cells, but not by serum, from vaccinated donors (Table 1), suggesting that it represents a cell-mediated type of immune response.

Histological examination of the liver confirmed that large numbers of cells accumulated in this organ during *P. yoelii* infection, especially in vaccinated mice; 7 days after infection, vaccinated mice showed more extensive cellular infiltration than did unvaccinated mice, in both the periportal area and the sinusoids. Very few cells were seen here in uninfected mice. Viable cells could be extracted from the liver by a combination of chopping and shaking; about four times as many ($15 \pm 3 \times 10^6$) were obtained from infected mice on day 7, and seven times as many ($22 \pm 4 \times 10^6$) from vaccinated-infected, as from uninfected control mice ($3.2 \pm 0.4 \times 10^6$). Most of these cells were lymphocytes, but vaccination also caused an increase in the absolute number of monocytes, blast cells and eosinophils. In contrast, vaccination had little effect on the numbers of any type of cell in the spleen at this time.

To test whether these cells infiltrating the liver could kill *P. yoelii* parasites, samples were incubated *in vitro* for 16 h with

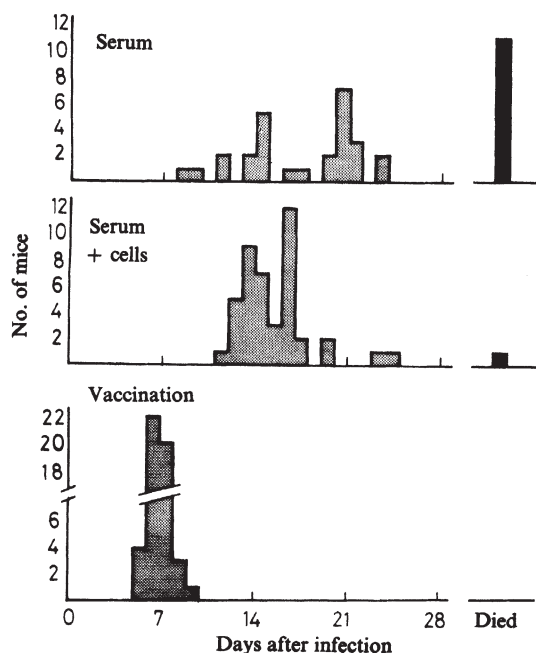


Fig. 1 Protection of mice against lethal *P. yoelii* infection by passively transferred cells and serum. Mice were given 1.5 ml of 'recovered serum' (see text) i.v. with or without 80×10^6 spleen cells from the same donors, either 2 h before infection with 10^4 *P. yoelii* or, in the case of serum, at various times after infection. Hatched columns represent the number of mice that recovered from infection, plotted against the day when they first cleared their parasitaemia (less than 1 parasite per 10,000 red cells). Solid columns represent mice that died. For comparison the survival times are also shown for 50 consecutively infected mice vaccinated 3 weeks previously with *P. yoelii* vaccine plus *B. pertussis*.

parasitised red cells, and the survival of parasites estimated by an *in vivo* assay⁸. The addition of 10^7 viable cells from the livers of vaccinated mice taken 7 days after infection, to 10^5 *P. yoelii*-parasitised red cells, resulted in more than 90% loss of infectivity, which is greater than that caused by 10^7 spleen cells from the same mice, or by liver or spleen from unvaccinated mice (Fig. 3). However by day 10, unvaccinated-infected liver cells were much more effective (<1% survival), which may explain the ability of mice given antiserum to recover at this time (Fig. 1). Results obtained with various cells from uninfected mice (Fig. 3) suggested that the killing activity is nonspecific and resides mainly in cells of bone marrow origin, and we propose

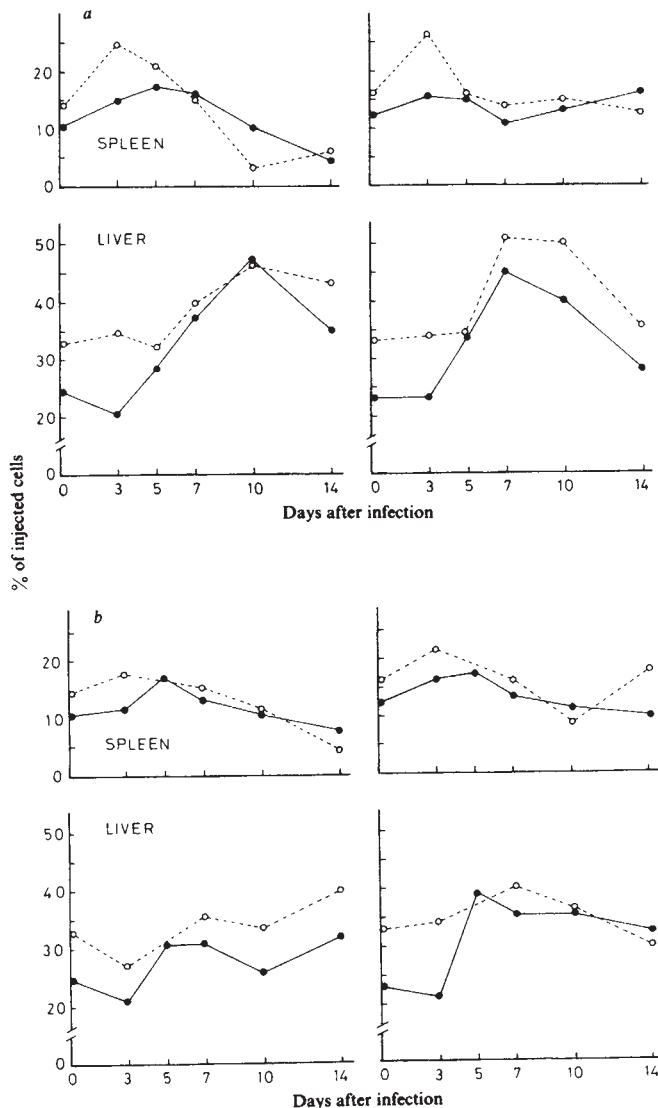


Fig. 2 *a*, Changes in the accumulation pattern of cells during lethal *P. yoelii* infection. 5×10^6 normal bone marrow (●) or lymph node (○) cells were labelled by incubating for 30 min at 37 °C with 100 μ Ci of ^{51}Cr as sodium chromate (Radiochemical Centre, Amersham), washed, and injected i.v. at various times after infection with 10^4 parasites. 24 h later the radioactivity in the recipient spleens and livers was counted in an LKB Wallac γ counter. The radioactivity per organ is expressed as a percentage of the total dose injected. Upper panels, accumulation in spleen; lower panels, accumulation in liver. Left, infected normal mice; right, infected mice vaccinated 3 weeks previously with *P. yoelii* vaccine plus *B. pertussis*. The values shown are the mean of 3–8 infected mice and 20–40 uninfected controls (day 0 values). s.e. (not shown) was usually less than 5% and always less than 10% of the mean. *b*, Changes in the accumulation pattern of cells during *P. berghei* infection. All details as for *a*, except that infection and vaccination were with *P. berghei*.

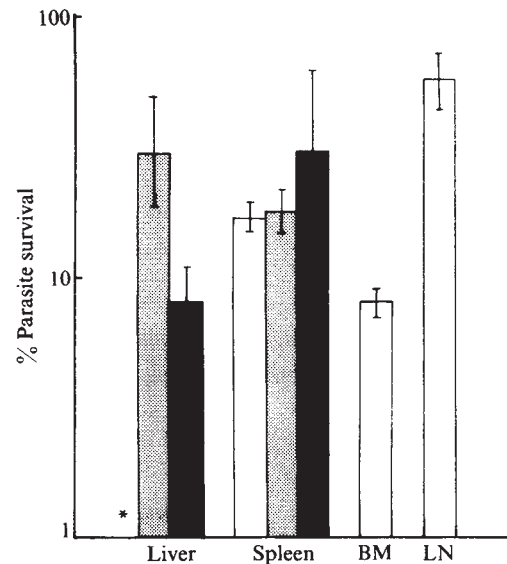


Fig. 3 Killing of *P. yoelii* by cells *in vitro*. Cultures consisting of 10^7 viable cells per ml in medium NCTC 135 containing 0.0001% ferric nitrate, 2,000 units of streptomycin, 1,000 units of penicillin, and 10–20% normal horse serum in 1 ml sterile plastic syringes (Sable Gillette Surgical) were mixed with parasitised red cells obtained from a mouse infected with non-lethal *P. yoelii* less than 7 days earlier, at a final concentration of 10^5 cells per ml. A supplement of 10^8 washed normal red cells was also included. After 16 h incubation at 37 °C, aliquots were injected i.v. into groups of outbred mice (Tuck's No. 1). Blood smears were made from the mice daily and stained with Giemsa, and the percentage of parasitised red cells determined. The time since injection by which 0.5% of red cells had become infected was estimated and this figure was converted to give the number of infective parasites originally injected by reference to a standard calibration curve. The curve had been prepared by plotting the mean time for the parasitaemia to reach 0.5% in large groups of mice injected with a range of parasites from 10^0 – 10^6 (ref. 8). The values shown are the mean ± 1 s.e. of the log-transformed data. Open columns, control, uninfected mice. Hatched columns, infected unvaccinated mice. Solid columns, infected mice vaccinated as described in Fig. 2*a*.

* Insufficient cells obtained from normal liver.

that it is the attraction of these into the liver which underlies its effectiveness. We have not yet identified the responsible cell, but the monocyte, the neutrophil and the eosinophil are possible candidates (J.T., H.M.D. and J.H.L.P., in preparation). The accumulation of lymphocytes in the liver (Fig. 2) is interesting, since normal lymphocytes have little cytotoxic effect (Fig. 3). But it must be remembered that during infection, and particularly after vaccination, many infiltrating lymphocytes, both T and B, will be specifically primed, and there might also be increased numbers of 'natural' killer (NK) cells. The appearance of 'crisis forms' just before recovery, as described by others^{5,9}, seems to rule out phagocytosis as the primary killing mechanism, and this is supported by the finding that silica, which destroys phagocytic cells *in vivo*, did not prevent recovery in vaccinated mice².

The role of antibody in vaccinated mice is not clear. Addition of high-titre antiserum to our *in vitro* cultures can result in up to 90% further loss of infectivity, and it is possible that in the close vicinity of the antibody-forming cells, killing is even more efficient. Our experiments suggest the liver as a place where a high concentration of antibody, effector cells and parasites may occur; the major role of the spleen would then be to provide the antibody-forming cells. Accumulation of plasma cells has been noted in both spleen and liver of malarious mice by other workers¹⁰. Note that we have studied only the blood stage of the disease; naturally infected animals that have recently undergone the liver stage of infection might have a modified cell population

in their livers, and we are now investigating whether or not this results in a different response to blood-stage vaccines.

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Specific *in vitro* antibody response to influenza virus by human blood lymphocytes

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Advances in understanding of human immune responses depend, for obvious reasons, on the use of *in vitro* techniques for culture of peripheral blood lymphocytes (PBL). We report here that specific antibody responses to influenza virus can readily be obtained using simple, reproducible methods. The system will be useful for the analysis of the cellular requirements for antibody production, the genetics of immune responsiveness and in clinical studies of immunosuppressed and immunodeficient patients.

Most of the work done so far has been with the polyclonal activator, pokeweed mitogen (PWM), but this system, although it has yielded many interesting results^{1,2}, is less satisfactory than antigen specific responses. For example, some of the cell interactions bypass requirements (such as histocompatibility) known from murine work to be necessary for T–B cell collaboration in specific antibody production³. A satisfactory method for specific *in vitro* antibody production by human blood lymphocytes, has, however, proved very difficult to achieve. Several systems have been reported⁴ including responses to particulate (sheep red cells and DNP-polyacrylimide beads) and soluble (tetanus toxoid and ovalbumin) antigens but these either rely on the addition of factors obtained from activated cells⁵ or depend on difficult PFC (plaque forming cell) techniques which may be susceptible to artefacts⁶. Moreover, histocompatibility between cooperating cell populations is apparently not required for any of these responses. Finally, sheep red blood cells (SRBC), which are frequently used for human antibody responses, may be

Table 2 T cell dependency of human *in vitro* anti-influenza antibody response

Cells	Stimulus	Response	
		Anti-X31 (c.p.m.)	HIg ($\mu\text{g ml}^{-1}$)
PBL	X31	1,940 $\pm$ 332	<0.2
	PWM	1,500 $\pm$ 640	86 $\pm$ 21
E+	X31	98 $\pm$ 31	<0.2
	PWM	89 $\pm$ 29	<0.2
E–	X31	268 $\pm$ 35	<0.2
	PWM	67 $\pm$ 95	<0.2
E– + E+	X31	1,000 $\pm$ 246	<0.2
	PWM	1,829 $\pm$ 190	89 $\pm$ 22
E– + irradiated E+	X31	104 $\pm$ 15	<1.00
	PWM	2,100 $\pm$ 358	106 $\pm$ 19
E– + allogeneic E+	X31	296 $\pm$ 25	ND
	PWM	ND	94 $\pm$ 18

PBL prepared on Ficoll–Hypaque gradients were suspended to 10^7 cells per ml in RPMI 1640 containing 25 mM HEPES and 10% FCS and mixed with two vols of 2% neuraminidase-treated SRBC. The cells were then centrifuged into a pellet (400g for 15 min) and incubated at room temperature for 60 min to form rosettes. Cells forming rosettes (E+) were separated from non-rosette forming cells (E–) by centrifugation through Ficoll–Hypaque (density 1.08 g ml^{-1}). Red cells were removed from the E+ fraction by hypotonic lysis. Both E– and E+ fractions were cultured at cell concentrations equivalent to their proportions in unseparated PBL. These were approximately 0.5×10^6 for E– and 1.5×10^6 for E+. Increasing these concentrations up to 2×10^6 did not increase the response of the unseparated cells to either X31 or PWM. Irradiated cells received 5,000 rads from a therapeutic 60°C source. ND, not determined.

inappropriate as an antigen for analysis of cell interactions since SRBC-specific B cells bind to SRBC by their surface receptors and are found exclusively in the E+ (T) cell fraction following T–B cell separation by rosette formation⁶. By contrast, the method described here for specific *in vitro* antibody production by human blood lymphocytes (PBL) to influenza virus is simple and reproducible, does not require special conditions, is T cell-dependent, requires histocompatibility between cooperating E rosette negative (E–) and E rosette positive (E+) cell fractions and, above all, uses a clinically relevant antigen. Careful analysis of this system showed that immune complexes formed between the stimulating antigen and antibody present in serum used to supplement cultures were a major source of pseudo antibody-formation. This artefact was completely avoided by the use of horse rather than human serum together with detection of anti-influenza antibody produced in culture by radioimmunoassay specific for human immunoglobulin (Ig). In this way, the RIA was superior to PFC assays which cannot distinguish between human and non-human (such as horse) complement fixing antibody.

Figure 1 shows the human blood lymphocyte antibody response to influenza virus strain A/X31 obtained from supernatants prepared at various times after initiation of the culture. A maximal response was obtained at day 6 which is consistent with the polyclonal antibody response to PWM¹. Specificity of the response was demonstrated by culturing cells with two non-cross reacting strains of flu virus, X31/A and HK/B. Antibody produced in cultures stimulated with X31 was specific for X31 and did not bind to HKB in the radioimmunoassay (Table 1). Similarly antibody elicited in cultures with HKB virus was specific for HKB and did not bind to X31. Further evidence for the specificity of this response was obtained by comparing antibody specific for X31 or HKB with total Ig production in cultures stimulated with X31, HKB or PWM. It was found that PWM and influenza virus generated similar amounts of specific anti-influenza antibody, whereas significant total Ig production occurred only in PWM cultures (Table 1). This finding shows that anti-X31 antibody formed in PWM cultures is only a small part of a polyclonal response. By contrast, the failure to detect a significant increase in total Ig production in cultures stimulated with virus indicates that this response is restricted to a small proportion of the total possible clones.

The response to X31 was shown to be dependent on metabolic activity and protein synthesis by the addition of either sodium

Table 1 Human *in vitro* anti-influenza antibody response

Stimulus	Inhibitor	Anti-X31 (c.p.m.)	Anti-HKB (c.p.m.)	HIg ($\mu\text{g ml}^{-1}$)
None	—	345 $\pm$ 146	39 $\pm$ 11	<0.2
X31	—	3,703 $\pm$ 886	47 $\pm$ 15	<0.2
HKB	—	203 $\pm$ 257	1,538 $\pm$ 408	<0.2
PWM	—	1,159 $\pm$ 283	563 $\pm$ 153	57 $\pm$ 8
X31	Cycloheximide	75 $\pm$ 56	39 $\pm$ 14	ND
X31	Sodium azide	42 $\pm$ 28	ND	ND

Human PBL prepared on Ficoll–Hypaque gradients were cultured at a concentration of 2×10^6 viable cells per ml with either X31 ($5 \mu\text{g ml}^{-1}$), HKB ($2 \mu\text{g ml}^{-1}$) or PWM (1:100) in the presence of 10% horse serum. After 6 days, supernatants were collected and assayed for specific anti-viral antibody and for total human Ig. Antibody synthesis was completely inhibited by the presence of cycloheximide ($2.5 \mu\text{g ml}^{-1}$) or sodium azide (0.05%) during the final 24 h of culture. ND, not determined.

azide or cycloheximide. In each case, addition of the inhibitor completely abrogated the response to X31 (Table 1).

T cell dependency of the human anti-influenza response was demonstrated by E-rosette separation of T and B cells. As shown in Table 2, neither E+ or E- cells alone responded to X31 whereas combined E- and E+ populations responded almost as well as unseparated PBL. Moreover, the polyclonal response to PWM also required T cells confirming the findings of other workers⁷. However, whereas irradiated E+ cells could provide help in the PWM response, they were unable to collaborate with B cells in the specific antibody response to X31 (Table 2). A further distinction between the specific response to X31 and PWM was the requirement of histocompatible E- and E+ cell populations (Table 2). This finding is predicted from work with experimental animals and strongly supports the contention that the response to X31 is a genuine antibody response.

In contrast to the above clearcut results, obtained from cultures supplemented with horse serum, the use of human serum created artefacts which obscured the specific antibody response to X31. Cultures with both X31 and PWM were performed with PBL from the same donor and under conditions identical to those described above except that human AB serum from a source which had been shown previously to support PWM response was used instead of horse serum and cells were washed five times just before the second incubation in order to remove contaminating human serum. As shown in Table 3, human anti-X31 antibody was detected in culture supernatants which both required and was specific for the stimulating virus. However, treatment with either cycloheximide or sodium azide failed to decrease the response below 60% of untreated cultures. In addition, separation into E+ and E- fractions did not abrogate the response. In fact, more anti-X31 antibody was detected in E+ cultures than in cultures of unfractionated cells. Anti-X31 antibody produced by E+ cells was also insensitive to cycloheximide. There are three possible explanations for this apparent T cell antibody response to X31. First, sufficient B cells may have contaminated the E+ fraction to permit antibody production. Indeed, fluorescent labelling for surface Ig detected approximately 1% surface Ig+ (B) cells in the E+ fraction. However, when these few remaining B cells were removed by rosetting with ox red cells coated with affinity purified goat anti-HiG⁸, the E+ and PBL response to X31 was unaffected. The ability of this rosetting method to remove all Ig+ (B) cells was demonstrated by the absence of Ig+ cells and the complete abrogation of the antibody response to PWM. Second, E+ cells may have been producing a T cell product carrying v-region immunoglobulin determinants detected by the ¹²⁵I-labelled goat

anti-human Ig used in the radioimmunoassay. The anti-X31 activity in E+ cultures was, however, also detected with ¹²⁵I-goat anti-human Fc and ¹²⁵I-sheep anti-human μ and γ heavy chain antibody. This would suggest that E+ cells were producing complete IgM and IgG and was considered extremely unlikely. Third, it was possible that virus added to cultures with human serum (containing anti-influenza antibody) would form immune complexes which would bind to Fc receptors on B cells, monocytes and T cells. After washing, these complexes may elute off resulting in pseudo antibody production. In favour of this explanation were the following findings: (1) The protein synthesis inhibitor, cycloheximide, did not abrogate the response to X31 in human serum. By contrast, PWM stimulated anti-X31 antibody production was lost following cycloheximide treatment (Table 3). (2) Surface Ig+ cells could be detected, with FITC-F(ab)₂ goat anti-human Ig, at day 6 in cultures of E+ cells stimulated with X31 in the presence of human serum but not horse serum. (3) Pseudo anti-X31 antibody production by E+ cells required both human serum (containing anti-X31 antibody) and specific antigen in culture. That is, no X31 antibody was detected by RIA (specific for human Ig) in cultures of E+ cells stimulated with X31 in the presence of horse serum (Table 2) or in human serum in the absence of specific antigen.

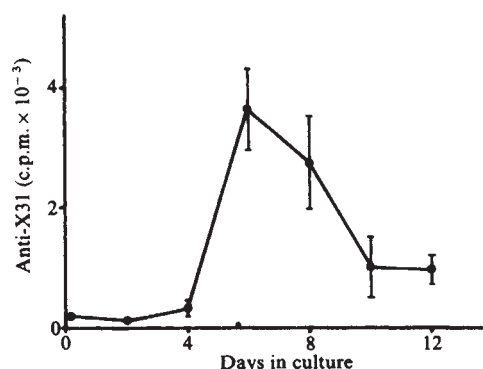


Fig. 1 Time response to X31. Human PBL were cultured with influenza virus essentially as described by Fauci *et al.* for PWM activation⁹. Briefly, human blood mononuclear cells were prepared from Ficoll-Hypaque gradients, washed and cultured at a concentration of 2×10^6 cells per ml in 12×75 mm capped Falcon tubes in medium RPMI 1640 (Gibco Biocult) containing 2 g l^{-1} NaHCO₃, 25 mM HEPES, 10^{-5} M hydrocortisone and 10% horse serum. Purified influenza virus strains X31 (A-H₃N₂) and HKB (B-8/73) (supplied by Dr Skehel, MRC, Mill Hill) were added at a final concentration of $5 \mu\text{g ml}^{-1}$ and $2 \mu\text{g ml}^{-1}$ respectively. These concentrations were shown to be optimal in preliminary experiments. The cultures were incubated at 37 °C for 6 days in an atmosphere of 5% CO₂ in air. At the completion of the culture period, the cells were washed twice, resuspended in 0.5 ml of RPMI 1640 containing 25 mM HEPES and 5% fetal calf serum (FCS, Flow Laboratories) and incubated for a further 20 h at 37 °C. Culture supernatants (SN) were then removed and stored at -20 °C before RIA for anti-influenza antibody. For the RIA, virus at a concentration of $100 \mu\text{g ml}^{-1}$ was adsorbed onto flexible plastic microtitre plates (Gibco Biocult) for 1 h at 37 °C. The plates were then washed three times and any further nonspecific binding sites blocked by incubation with 5% FCS in BSS (balanced salt solution). After two washes, 40 μl of culture supernatant was added to each well, incubated for 1 h at 37 °C, and washed three times. Finally, 50 μl containing 50,000 c.p.m. of ¹²⁵I-labelled immunoabsorbent purified goat anti-human Ig (¹²⁵I G α HiG, Sp. activity $5 \mu\text{Ci } \mu\text{g}^{-1}$) was added for a further 1 h at 37 °C. After unbound ¹²⁵I was removed by extensive washing, the plates were cut up with scissors and each well counted for bound radioactivity in a Wallac LKB 1280 ultragamma counter. Results were expressed as mean c.p.m. $\pm$ s.e. obtained from triplicate cultures. Backgrounds determined by replacing the second layer (culture SN) with 5% FCS in BSS were usually below 200 c.p.m. and were subtracted from experimental values. Further controls using other antigens such as FCS, or BSA adsorbed to the plate were likewise, always below 200 c.p.m. A similar technique was used to determine total HiG produced in PWM cultures except a competition rather than a direct assay was used. Briefly, 25 μl of culture supernatant and 10 μl ¹²⁵I G α HiG (40,000 c.p.m.) were incubated for 1 h at 37 °C and then added to plates previously coated with HiG in the same fashion as described for X31. After incubation for 1 h at 37 °C the plates were washed and bound activity determined. The concentration of HiG present in each supernatant was determined by comparison with a standard curve obtained from known concentrations of human Ig.

Table 3 Apparent human anti-influenza antibody production obtained from cultures containing human serum

Cells	Stimulus	Inhibitor	Response		
			Anti-X31 (c.p.m.)	Anti-HKB (c.p.m.)	HiG ($\mu\text{g ml}^{-1}$)
PBL	X31	—	$2,173 \pm 861$	208 ± 129	<1
	HKB	—	234 ± 84	$1,162 \pm 413$	<1
	PWM	—	679 ± 91	616 ± 85	40 ± 3
PBL	X31	Cycloheximide	$1,217 \pm 186$	ND	ND
	PWM	Cycloheximide	90 ± 6	ND	1.0 ± 0.1
E+	X31	—	$2,850 \pm 299$	ND	<0.2
E+	PWM	—	97 ± 29	ND	<0.1
E+	X31	Cycloheximide	$1,875 \pm 184$	ND	ND
E-	X31	—	$1,006 \pm 110$	ND	ND
E- + E+	X31	—	$1,072 \pm 116$	ND	ND

Human PBL prepared on Ficoll-Hypaque gradients were cultured at a concentration of 2×10^6 viable cells per ml with either X31 ($5 \mu\text{g ml}^{-1}$), HKB ($2 \mu\text{g ml}^{-1}$) or PWM (1:100) in the presence of 10% human serum. After 6 days in culture, cells were washed five times and incubated for a further 20 h in RPMI 1640 containing 25 mM HEPES and 5% FCS. Supernatants were then collected and assayed for specific anti-viral antibody and for total human Ig. In the presence of human serum, cycloheximide ($2.5 \mu\text{g ml}^{-1}$) did not reduce the anti-X31 antibody response below 60% of untreated cultures but did abrogate both the anti-X31 and total Ig response to PWM. E+ and E- cells were prepared and cultured as described in Table 2. ND, not determined.

The presence of antibody-antigen complexes on the cell surface at the end of the culture period, not removed by up to five washes, could also result in pseudo plaque formation if the complexes were slowly released from the cell surface during incubation, although the plaques would necessarily be very small. In the system described here, this artefact was obviated by the use of horse serum in the culture and radioimmunoassay specific for human Ig produced in culture to the stimulating antigen.

The development of an *in vitro* system for specific antibody production by human PBL which both is T-dependent, and appears to require genetic compatibility between cooperating cell populations opens the way for a number of studies which have not hitherto been possible. For example, the role played by HLA-D region in cell interactions required for antibody production can now be examined. Furthermore, although most people have been exposed in nature to the influenza strains used in this work (that is, the responses measured are secondary), a significant proportion of individuals tested did not make antibody *in vitro*. It is, therefore, feasible to test for association between HLA phenotype and immune responsiveness either prior to or following immunisation with available vaccines. Finally, the use of clinically relevant antigens makes it possible to study associations between immune deficiencies and some other diseases (such as subacute sclerosing panencephalitis) with altered antibody responses which can be measured *in vitro*.

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Glucocorticoid hormones inhibit DNA synthesis and enhance interferon production in a human lymphoid cell line

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Although buffy coat leukocytes have been the prime source of human interferon, cells of the Burkitt lymphoma line Namalwa are increasingly used for the large scale production of interferon¹⁻³. On induction with Sendai virus or Newcastle disease virus, Namalwa cells produce a substantial quantity of interferon which contains predominantly the leukocyte antigenic species and minor amounts of fibroblast-type interferon^{4,5}. We have recently demonstrated that inducers of erythropoietic differentiation in Friend cells are able to enhance interferon synthesis in Namalwa cells when added to cultures ≥ 24 h before interferon induction by Sendai virus⁶. The most potent compound, *n*-butyrate, stimulated interferon production about 30-fold and has also been independently described by others⁷. All active compounds inhibited DNA synthesis in Namalwa cells and the extent of inhibition apparently paralleled the stimulatory potency of the respective compound. Induction of differentiation of Friend cells can be antagonised by various steroid hormones, which by themselves have no measurable effects on these cells^{8,9}. In contrast, we report here that glucocorticoid hormones inhibit DNA synthesis in Namalwa cells and augment Sendai virus-induced interferon synthesis.

Table 1 Effects of steroids on DNA synthesis and interferon production of Namalwa cells

	Interferon titre		Inhibition of DNA synthesis (%)
	Range (units per 10 ⁶ cells)	Proportional increase Range Mean	
Dexamethasone	8,000-36,000	11-33 26	87 $\pm$ 7
Triamcinolone	1,400-16,000	19-22 21	86 $\pm$ 10
Hydrocortisone	2,400-42,000	8-50 22	82 $\pm$ 14
Cortisone	320-1,080	1-3 2	56 $\pm$ 11
Prednisolone	11,000-31,000	15-36 26	87 $\pm$ 7
Prednisone	460-1,270	1-4 3	38 $\pm$ 8
Aldosterone	290-620	3-4 3	16 $\pm$ 12
Cortisolone	860-1,610	2-4 3	-4 $\pm$ 5
17 β -Oestradiol	390-400	0.5-1 1	45 $\pm$ 6
Testosterone	320-1,050	1 1	10 $\pm$ 20
Progesterone	500-2,160	1-2 2	90 $\pm$ 5
Etiocanolone	500-580	1 1	0 $\pm$ 1

Cells were cultured in Eagle's minimum essential medium (Earle's salts) containing double amounts of vitamins and amino acids, 10% fetal calf serum (GIBCO), 100 U ml⁻¹ penicillin G and 50 U ml⁻¹ streptomycin at 37 °C in a humidified atmosphere containing 5% CO₂. Suspensions of rapidly growing cells were diluted to 5 $\times$ 10⁵ cells per ml with fresh medium and distributed into plastic culture flasks (10 ml per flask). The compounds to be tested were added at 10 μ M (steroid stocks were prepared using absolute ethanol); each experiment included untreated control cultures. After 48 h, DNA synthesis was measured by incorporation of tritiated thymidine: 0.5-ml aliquots of the cell suspension were incubated with 3 μ Ci of [Me-³H]thymidine (47.5 Ci mmol⁻¹, Radiochemical Centre) for 15 min at 37 °C. The radioactivity incorporated into acid-precipitable material was determined by liquid scintillation counting. For induction of interferon synthesis, the cells were collected by centrifugation after 48 h, washed with medium, suspended at 10-15 $\times$ 10⁶ cells per ml in medium containing 2% fetal calf serum and 2¹⁰ haemagglutinating units Sendai virus per ml and incubated at 37 °C for 2 h with constant shaking. The cells were then pelleted, resuspended in serum-free medium at cell densities of 1-1.5 $\times$ 10⁶ cells per ml and incubated for 20 h at 37 °C. Cell supernatants were collected, adjusted to pH 2 with HCl and kept at 4 °C for 4 d to inactivate residual inducer virus. Interferon titres were defined as the reciprocal of the cell supernatant dilution causing a 50% reduction of the plaque count of vesicular stomatitis virus on V3 cells. All titres were expressed in terms of the reference standard 69/19. The actual interferon titres as well as the increase in interferon production in treated cells over that in untreated control cells of the same experiment are shown. All steroids were tested at least three times in independent experiments. DNA synthesis in steroid-treated cultures is expressed relative to that of control cultures of the respective experiment (mean and standard deviation); control cells incorporated 4-6 $\times$ 10⁴ c.p.m. per 10⁶ cells.

In preliminary experiments, we observed that hydrocortisone did not antagonise the effects of *n*-butyrate on cell proliferation and interferon synthesis in Namalwa cells, as it had done in the Friend cell system. Surprisingly, however, hydrocortisone by itself prevented Namalwa cell growth at molar concentrations 10⁴-fold lower than the effective concentration of butyrate. This was paralleled by a marked enhancement of interferon production after Sendai virus induction (Table 1, Fig. 1). We consequently surveyed several structurally different natural and synthetic steroids with respect to their effect on Namalwa cell growth and interferon synthesis. As a standard procedure, cells were cultured for 48 h in the presence of a steroid at 10 μ M. DNA synthesis was determined by measuring the incorporation of labelled thymidine into acid-precipitable material; interferon synthesis was induced by Sendai virus and its activity established in a virus plaque reduction assay. The results are summarised in Table 1. The glucocorticoids dexamethasone, triamcinolone, hydrocortisone and prednisolone stimulated interferon production more than 20-fold, whereas a mineral corticoid like aldosterone or sex steroids showed only weak or no enhancing activity.

Structurally, an 11 β -hydroxyl group seems to be necessary for activity, as all glucocorticoids which markedly stimulate

interferon production contain this configuration; its replacement by a keto group, as in cortisone and prednisone, is accompanied by an extensive loss in activity. All glucocorticoids which elevated interferon production diminished DNA synthesis and stopped proliferation of Namalwa cells without affecting cell viability, but no causal relationship can be deduced from this correlation. Within 24 h after transfer of the cells to medium without glucocorticoids, DNA synthesis returned to the level of untreated cells and cell densities had doubled after 48 h. We found the only exception to this reciprocal link to be progesterone, which blocks DNA synthesis with no accompanying effect on interferon production. No compound, however, has yet been found to enhance interferon synthesis without concomitant inhibition of DNA synthesis (an observation possibly valuable for screening stimulating substances) whereas the reverse relationship does not hold true—arrest of Namalwa DNA synthesis by a variety of methods fails to lead to elevated interferon titres.⁶

Dose-response relationships of the four glucocorticoids with strong stimulatory power are shown in Fig. 1. They emphasise the close correlation between glucocorticoid effects on DNA synthesis and interferon production, and imply that the same cellular receptor is mediating both actions. At steroid concentrations of 1–10 μM , enhancement of interferon production and inhibition of DNA synthesis reach their highest level; at 0.1 μM the effects are semi-optimal. A slight stimulation of DNA synthesis occurring at low glucocorticoid concentrations (1–10 nM) was not examined in more detail. When Namalwa cells were exposed to the 'anti-glucocorticoid' corticosterone^{10,11} (which at 10 μM had no significant effect on DNA synthesis and only marginally affected interferon production) together with 0.1 μM dexamethasone, the action of the latter steroid was partially abolished: inhibition of DNA synthesis was reduced from 78% to 45% and augmentation of interferon production

diminished by more than 60%. These results, together with the relative potencies of the glucocorticoids (Fig. 1), suggest the involvement of a typical glucocorticoid receptor.

Time course studies show that the glucocorticoid action is a slow process (Fig. 2); inhibition of DNA synthesis was only marginal 10 h after treatment with 1 μM dexamethasone, and reached a half-maximal level after 17–18 h. In accordance with these data, we observed that cell densities of the cultures approximately doubled after addition of dexamethasone and remained constant thereafter. Enhancement of interferon synthesis attained half-maximal values after 24 h and thus lagged behind the arrest of DNA synthesis.

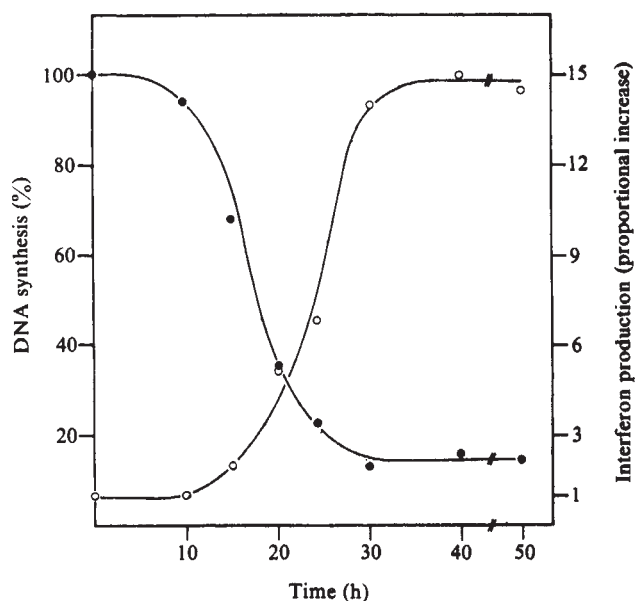


Fig. 2 Kinetics of dexamethasone action on DNA synthesis and interferon production. A culture of rapidly growing Namalwa cells was diluted to 6×10^5 cells per ml and distributed into culture flasks (10 ml per flask). 1 μM dexamethasone was added for various time periods before measurement of DNA synthesis and induction of interferon production (see Table 1 legend). All points represent the means of duplicate cultures; results were confirmed in two further independent experiments. ●, DNA synthesis of steroid-treated relative to untreated cells. ○ Relative increase in interferon synthesis (mean titre of control cultures ± 1 corresponding to 310 reference standard units per 10^6 cells).

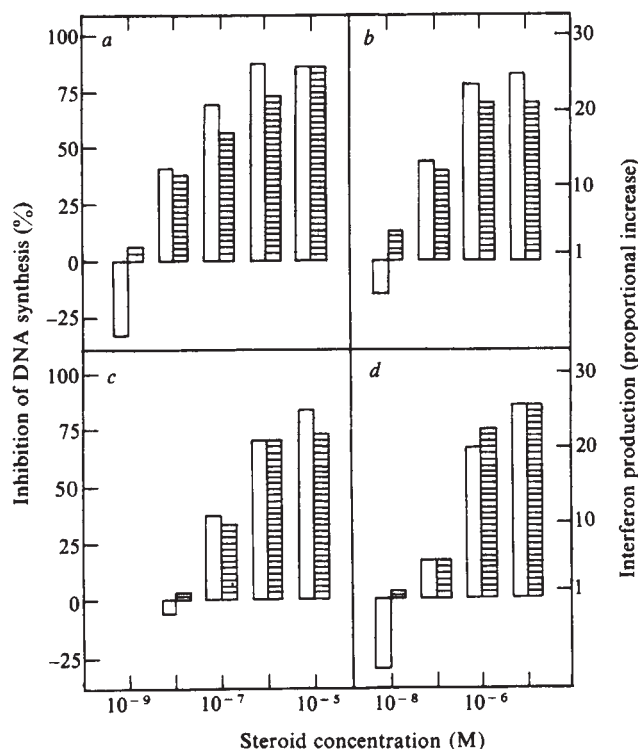


Fig. 1 Glucocorticoid action on DNA synthesis and interferon production: dose-response relationships. Treatment of cells with glucocorticoids (48 h), determination of DNA synthesis, induction and assay of interferon were essentially as described in Table 1 legend. Inhibition of DNA synthesis (open bars) is given relative to untreated, parallel controls (untreated cells incorporated $4-6 \times 10^4$ c.p.m. per 10^6 cells); interferon production (hatched bars) is displayed in relative units (untreated cultures = 1). a, Dexamethasone; b, triamcinolone; c, hydrocortisone; d, prednisolone.

Concomitant exposure of cells to butyrate and dexamethasone revealed that the glucocorticoid did not antagonise the interferon stimulating activity of butyrate but also did not further enhance interferon yields when optimal butyrate concentrations were applied. Both classes of substance were synergistically active, however, when suboptimal doses were used. These results suggest that both fatty acids and glucocorticoids may interact with the same mechanism which finally leads to enhanced expression of interferon and block of DNA synthesis, although not necessarily at the same stage. Our results provide further evidence that the expression of the interferon gene(s) in human lymphoid cells is influenced by substances affecting cell differentiation in several *in vitro* systems. Interferon synthesis, however, is not immediately triggered by butyrate or corticosteroids, but is subjected to an additional control mechanism and still requires the presence of an inducing virus.

At present the advantages of the use of a continuous cell line for the production of human leukocyte interferon are limited by the comparatively modest yields. The marked stimulation of interferon synthesis by butyrate and glucocorticoid hormones may therefore be of considerable interest for the large scale production of human interferon.

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Shear-induced concanavalin A agglutination of human erythrocytes

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The mechanism by which cell suspensions are agglutinated by plant lectins remains obscure. The agglutination of a particular cell line in the presence of a specific plant lectin probably depends on several factors including the number and valence of lectin molecules bound to the cell surface, the mobility of receptor molecules in the membrane, the surface morphology and charge and the metabolic state of the cell^{1–6}. The assay system used to assess cell agglutination also seems to be important, since many laboratories studying the same agglutination reaction have reported dissimilar or contradictory results^{3,4,7}. To provide further information on the molecular mechanism of agglutination we have begun a systematic study on the aggregation of human red cells by the lectin concanavalin A (Con A). By using an adaptation of our previously described aggregation assay⁸ which provides a continuous measure of both the rate and degree of intercellular adhesion in the presence of controlled shear forces, we have found that the agglutination of erythrocytes by Con A can be resolved into three stages, two of which are observed only if the system is exposed to shear.

Our cell aggregation assay is based on the fact that if a fluid or cell suspension is subjected to a constant rate of shear in a viscometer, the shear stress (force per unit area) generated at the measuring element of the apparatus is proportional to the rate at which energy is dissipated throughout the sample during flow. For flow to occur in very concentrated suspensions intercellular associations, if present, must be broken down. Energy will necessarily be expended in this process. Other factors being equal, then, the shear stress recorded for an aggregated suspension will be higher than that for a disaggregated suspension. A quantitative index of the degree of aggregation present is provided by the ratio, R , of the shear stresses recorded for the aggregated and disaggregated suspensions of the same cell concentration and suspending medium viscosity at the same applied shear rate. We have used this assay to follow the development of Con A-induced agglutination in the viscometer as a function of time.

The agglutination reaction was initiated by the addition of concentrated Con A to a red cell suspension undergoing steady shear in a Couette viscometer (Fig. 1). The time development of the shear stress observed is dependent on Con A concentration, red cell concentration and shear rate. On addition of the lectin an immediate rise in shear stress is recorded. We have shown previously that the magnitude of this increase provides a quan-

titative index of the degree of aggregation present in the suspension⁸. The initial rise soon reaches a plateau defining what we term Type I aggregation. The same plateau value is reached if Con A is added to static suspensions and the aggregation allowed to develop for at least 60 min before shearing. However, we can demonstrate significant differences between static and Type I aggregation. As shearing proceeds a second marked increase in shear stress commences, in the case illustrated, 7 min after the addition of Con A. This increase denotes the onset of a second, stronger type of aggregation (Type II) which is induced by shear in the system. That this latter increase in shear stress represents a stronger type of aggregation and not, say, sedimentation can be demonstrated in several ways.

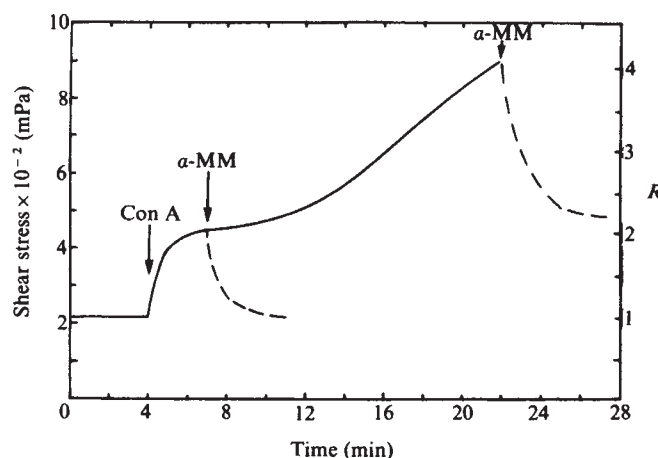


Fig. 1 The shear stress recorded as a function of time for aggregating erythrocytes. The right-hand axis shows the corresponding values for the index of aggregation, R . Fresh human red cells (ORh+) were washed four times in glucose-free, HEPES-buffered Hanks balanced salt solution, pH 7.4, suspended at a final microhaematocrit of 47% (5.1×10^9 cells ml^{-1}) and 0.9 ml placed in the cup of a Contraves LS-2 viscometer utilising a Couette measuring system and fitted with a custom-made guard ring to prevent the transmission of shear stresses at the liquid surface. A water circulator maintained the sample temperature at $37.0 \pm 0.1^\circ\text{C}$. At a constant shear rate of approximately 49 s^{-1} (~ 37 r.p.m. with the geometry used) a steady baseline shear stress, due to the viscosity of the unaggregated cell suspension, is obtained (apparent viscosity = shear stress/shear rate). Con A (Miles) is added where indicated in a volume of $23 \mu\text{l}$ to give an equilibrium solution concentration of $111 \mu\text{g ml}^{-1}$. The sample is then mixed by slowly raising and lowering the bob of the viscometer several times while shearing is maintained and the shear stress output recorded as a function of time. Type I aggregation is established within 2 min of Con A addition and the onset of Type II aggregation occurs approximately 4 min later. Microscopic examination of samples taken at various times showed that any non-aggregated cells retained their discoid shape and no haemolysis was observed under conditions where Type II aggregation had clearly formed. The dotted line at 3 min after Con A addition illustrates the shear stress behaviour if at that time the suspension is made 9.8 mM in $\alpha\text{-mm}$ by the addition of $10 \mu\text{l}$ of 500 mM $\alpha\text{-mm}$. If the addition of $\alpha\text{-mm}$ is delayed until 18 min after Con A addition, by which time Type II aggregation is well advanced, the behaviour indicated by the second broken line is observed. Including 9.8 mM $\alpha\text{-mm}$ in the medium before the addition of Con A results in complete inhibition of both Type I and Type II aggregation.

If the sample is mixed in the Type II region the shear stress recovers rapidly to its initial value. If the suspension is sampled at different times after adding Con A and another assay of aggregation, such as the erythrocyte sedimentation rate, is used to examine the system, the sample from Type II is found to be much more heavily aggregated than that from Type I, which in

turn is more aggregated than an unsheared suspension. Microscopic examination of Type I and II aggregated cells shows tightly clumped aggregates present in the former case and massive aggregates in the latter. Only moderate aggregation is visible in samples from static suspensions. If methyl α -D-mannopyranoside (α -mm) (9.8 mM), which is a competitive inhibitor of Con A binding, is added while Type I aggregation is present the shear stress returns to the level characteristic of the

disaggregated suspension. Microscopic examination of the system at this point confirms that Type I aggregation is fully reversed. Static aggregation is similarly reversed on addition of α -mm. In contrast, the same concentration of α -mm does not cause complete reversal of Type II aggregation. Figure 1 shows that if α -mm (9.8 mM) is added after the Type II stress increase has developed, the trace does not return to its pre-con A level but remains elevated. Microscopic examination confirms that aggregation is still present. Higher concentrations of α -mm (up to 55 mM) cause more extensive reversal of Type II (~80–90%) but complete reversal is not observed (data are not shown).

Type I and II aggregations can be further characterised by their response to increasing concentrations of Con A. While the R value for Type I aggregation rapidly saturates with Con A concentration (Fig. 2a), further increases decrease the lag time for the appearance of Type II and increase the rate at which the Type II shear stress develops (Figs 1 and 2b). The shear stress associated with Type II aggregation eventually levels off at an R value which is independent of Con A concentration (not shown). These data cannot be attributed to the kinetics of Con A binding since studies with ^{125}I -labelled Con A have demonstrated that the total amount of Con A associated with the cells (18.0×10^4 molecules per cell in the conditions of Fig. 1) is not measurably different in static, Type I or Type II aggregation. A more complex mechanism is also indicated by the α -mm reversal studies (Fig. 1). Using ^{125}I -Con A we find the same number of molecules bound per cell (4.0×10^4) after complete Type I reversal as after only partial Type II reversal.

Taken together these experiments suggest that Con A initially interacts in an α -mm sensitive fashion with cell surface oligosaccharides^{9,10}, causing static or Type I aggregation depending on whether or not the suspensions are under shear. Once Type I aggregation has been established, continued shearing allows a further change in the molecular bridging reaction causing the formation of a more stable cell-cell bond (Type II aggregation). This change presumably results from stressing the cell membranes in such a way that a finite number of new binding configurations are made available, resulting in a stronger intercellular bridge. These new binding configurations, responsible for Type II aggregation, may involve specific oligosaccharide sequences^{9–11}. Alternatively cell surface structures may interact with regions of the Con A molecule other than the carbohydrate binding site^{12,13}. In either case the interaction is less readily reversed by α -mm. At the cellular level these molecular events result in three types of aggregation, two of which (Types I and II) occur only in the presence of shear forces. We believe that these observations represent the first quantitative evidence that aggregation reactions can be resolved into three distinct stages¹⁴. Furthermore, they demonstrate that in addition to causing the breakdown of aggregates, shear forces can in some conditions induce considerably stronger aggregation than occurs in their absence.

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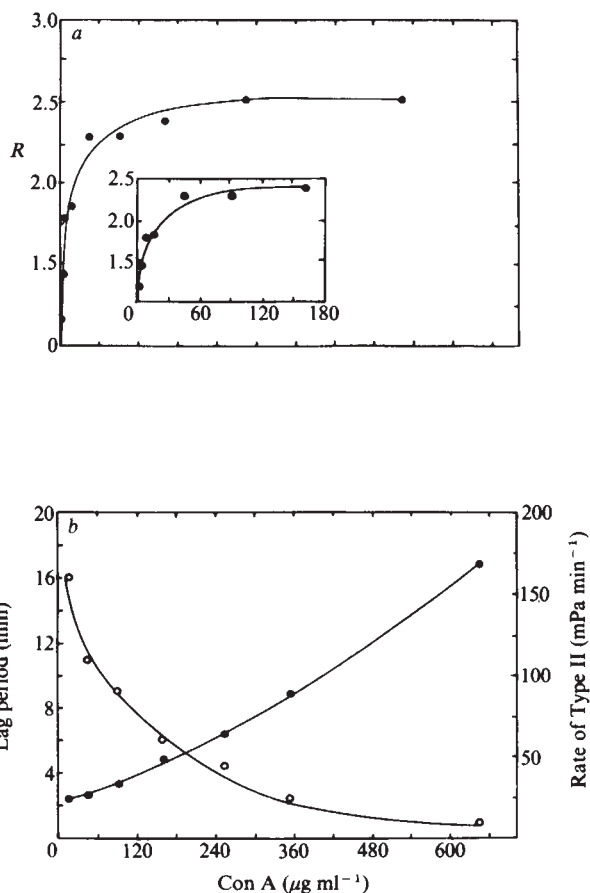


Fig. 2 a, The dependence of Type I aggregation on Con A concentration. Increasing concentrations of Con A were added to red cell suspensions in the conditions described in Fig. 1. The Type I R value associated with each tested concentration was calculated as described in the text and is expressed above as a function of the equilibrium Con A concentration. Estimation of the equilibrium Con A concentration was performed using ^{125}I -Con A. Iodination was carried out by a modification of Phillips and Furmanski's technique¹⁵ and the purity of the labelled product checked on 4% SDS-polyacrylamide gels. About 85% of the recovered counts co-migrated with the 27,000 protomer unit. Binding studies were performed by incubating red cell suspensions with ^{125}I -Con A (specific activity 2,500 c.p.m. μg^{-1}). At appropriate times samples were removed, spun for 15 s in an Eppendorf Model 3200 centrifuge and supernatant aliquots counted for ^{125}I . After correcting for the adsorption of radioactive material to the tube wall, the number of Con A molecules bound per cell was calculated from the activity lost from solution assuming a molecular weight for Con A of 1.1×10^5 (ref. 16). Including α -mm (100 mM) distinguishes between 'specific' and 'non-specific' binding. The insert shows the dependence of Type I R value at low Con A concentrations. b, The dependence of Type II aggregation on Con A concentration. Experimental conditions were as described in Fig. 1 and 2a. Progressive increases in the Con A equilibrium concentration shorten the lag period, defined as the time elapsed between the addition of Con A and the initial onset of Type II aggregation, and promote the rate at which the shear stress increases in Type II aggregation. This rate (slope of shear stress-time curve) (●) and the lag period (○) are plotted above as a function of the equilibrium Con A concentration.

Poly(ADP-ribose) levels in carcinogen-treated cells

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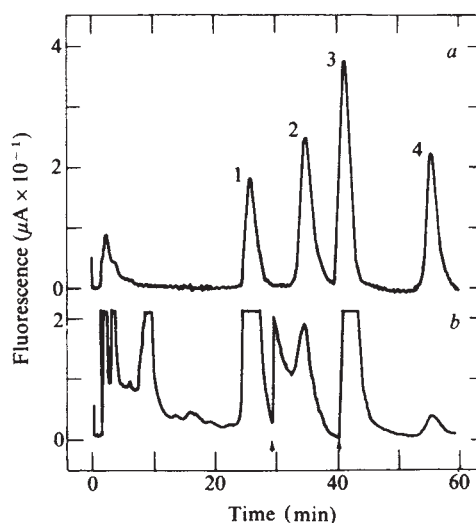
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Several reports indicate that intracellular levels of NAD are rapidly decreased by DNA-damaging agents such as streptozotocin¹⁻⁷, neocarzinostatin^{8,9} and ionising radiation⁸⁻¹¹. Similarly, evidence obtained from studies in isolated nuclei and permeabilised cells indicates that DNA damage caused by several alkylating chemicals¹²⁻¹⁵, X rays^{16,17} and DNA-hydrolysing enzymes^{18,19} elicit a considerable increase in the activity of poly(ADP-ribose) polymerase, a chromosomal enzyme which uses NAD as a substrate in the formation of poly(ADP-ribose) in histones and other nuclear proteins^{20,21}. Thus, it has been suggested that DNA-damaging agents lower NAD levels by causing an increase in poly(ADP-ribose) synthesis^{9,12,13}. As DNA damage initiates DNA repair mechanisms, it has also been argued that poly(ADP-ribose) is involved in DNA repair^{8,12,14,17,22,23}. Such possibilities would be strongly supported if an actual increase in the intracellular levels of poly(ADP-ribose) could be shown to occur after induction of DNA damage. We now present data obtained using a new technique which has enabled us to measure the levels of poly(ADP-ribose) *in vivo* in SV40 virus-transformed 3T3 cells (SVT2). We show

that treatment of these cells with the powerful mutagen *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (MNNG) produces, concomitantly with a decrease in NAD levels, a dramatic increase in the intracellular levels of poly(ADP-ribose).

The method used achieves separation of poly(ADP-ribose) from the bulk of RNA, DNA and proteins by affinity chromatography using dihydroxyboryl-Sepharose. Poly(ADP-ribose) is then treated with snake venom phosphodiesterase and bacterial alkaline phosphatase to yield the unique nucleoside 2'→1' ribosyladenosine from internal residues. Treatment with chloroacetaldehyde yields the fluorescent derivative, 1, *N*⁶-ethenoribosyladenosine (*ε*-ribosyladenosine), which is separated from other fluorescent compounds by reversed-phase HPLC, and picomole amounts are quantified by fluorescence detection. Figure 1 shows an HPLC profile obtained in a typical poly(ADP-ribose) determination. Chromatogram *a* shows the separation of the etheno derivatives of four adenine-containing compounds: *ε*-adenosine (peak 1), *ε*-ribosyladenosine (peak 2), *ε*-adenine (peak 3) and *ε*-2'-deoxyadenosine (peak 4). Chromatogram *b* shows the profile obtained when material equivalent to 4.3×10^7 SVT2 cells is chromatographed. Although relatively large amounts of material eluting in the position of *ε*-adenosine and *ε*-adenine are present, it is possible to visualise clearly a peak of *ε*-ribosyladenosine (peak 2). In addition to co-chromatographing with authentic *ε*-ribosyladenosine, peak 2 is not present when snake venom phosphodiesterase treatment is omitted from the procedure, when the samples are subjected to adenosine deaminase treatment before derivatisation with chloroacetaldehyde, or when chloroacetaldehyde treatment is omitted. When ¹⁴C-poly(ADP-ribose) of average chain length 2.5 or 20 is added to cell extracts and carried through the entire procedure, 60–70% of the radioactivity is recovered as the sum of *ε*-ribosyladenosine and *ε*-adenosine. The value obtained from SVT2 cells was 5 pmol per 10^8 cells of ribosyladenosine derived from poly(ADP-ribose).

Fig. 1 Determination of *in vivo* levels of poly(ADP-ribose). SVT2 cells were grown in Dulbecco's modified Eagle's media as described previously²⁷. Dihydroxyboryl-Sepharose was synthesised by the attachment of 6-amino-hexanoic acid to cyanogen bromide-activated Sepharose 4B followed by coupling of *m*-aminophenylboronic acid hemisulphate to the free carboxyl group of the spacer²⁸. Two Petri dishes, containing 50×10^6 SVT2 cells each, were set on ice. After aspiration of the media, cells were washed once with 5 ml of 0.01 M sodium phosphate buffer, pH 7.2, containing 0.21 M sodium chloride, and precipitated by the addition of 5 ml of cold 20% (w/v) trichloroacetic acid (TCA). After 20 min, the precipitate was scraped from the dishes with a rubber policeman and collected by centrifugation at 800g for 10 min. The pellet was washed twice with 20% TCA and twice with diethylether and suspended in 10 ml of 0.1 M potassium phosphate buffer, pH 5.0, containing 6 M guanidine hydrochloride. The pellets were subjected to sonification to facilitate solubilisation and the pH was adjusted to 8.5 with 6 M KOH. Affinity chromatography was carried out by applying the sample to a 35×4.5 mm dihydroxyboryl-Sepharose column (0.4 ml packed gel) pre-equilibrated with 0.1 M potassium phosphate buffer, pH 8.5, containing 6 M guanidine hydrochloride. After the sample, an additional 2 ml of the buffer was passed through the column to remove all unbound material. Guanidine and phosphate were removed by washing the gel with 5 ml of 10 mM sodium morpholine propane sulphonic acid (MOPS) buffer, pH 8.5. At this point, 200 μ l of 90 mM MOPS buffer, pH 8.5, containing 20 mM MgCl₂, 240 μ g of bacterial alkaline phosphatase and 75 μ g of snake venom phosphodiesterase, was injected directly into the gel in the column. After gentle stirring of the column to ensure good distribution of the enzymes, digestion was carried out by incubating the column, which had been sealed at both ends, at 37 °C for 3 h. Nucleosides were eluted from the gel with four 200- μ l aliquots of 20 mM sodium citrate buffer, pH 4.5. Protein was removed from the digests by adjusting them to 20% in TCA. Supernatants were extracted five times with an equal volume of diethylether and the volume was reduced to 300 μ l by aspiration *in vacuo*. The cell extract was then adjusted to 200 mM in sodium citrate buffer, pH 4.5 and 20 mM in chloroacetaldehyde. Fluorescent etheno derivatives were formed by incubating this mixture at 60 °C for 8 h. The samples were extracted five times with an equal volume of diethylether to remove chloroacetaldehyde. *ε*-Ribosyladenosine was separated from other fluorescent compounds by HPLC on a Micromeritics 7000B liquid chromatograph equipped with a Partisil PXS 10/25 ODS reversed phase column (Whatman) 250 mm $\times$ 4.6 mm i.d. $\times$ 5 mm o.d. Fluorescence was detected with a Schoeffel FS-970 fluorometer. The fluorometer excitation monochromator was set at 312 nm, a 7–54 excitation filter and a 398-nm emission filter were used, the sensitivity of the fluorometer was set at 64, and a time constant of 10 s used. This allows the detection of ethenoadenine compounds but not of ethenocytosine compounds. After sample injection, elution was carried out isocratically with 7 mM ammonium formate buffer, pH 5.8/100% methanol 97.5/2.5 (v/v). The flow rate was 1.33 ml min⁻¹ and the column temperature 50 °C. Chromatogram *a*, injection of 50 μ l containing the standards: *ε*-adenosine (1), *ε*-ribosyladenosine (2), *ε*-adenine (3) and *ε*-2'-deoxyadenosine (4). Chromatogram *b*, injection of 150 μ l of cell extract equivalent to 4.3×10^7 cells. Peak 2 in *b* represents 5 pmol ribosyladenosine per 10^8 cells. This value was corrected for overall recovery which was 65% for this particular experiment. The fluorometer recording range was changed from 1.0 μ A full scale to 0.1 μ A full scale in *b* in the region between the arrows.



When SVT2 cells are treated with 0.3 mM MNNG for 20 min, poly(ADP-ribose) levels increase dramatically (Fig. 2a) to a value of 750 pmol per 10^8 cells. This represents a 150-fold increase in the levels of poly(ADP-ribose). In the chromatograms of Fig. 2, the equivalent of only 5.4×10^6 cells was injected. This amount of sample will not show a measurable peak of ϵ -ribosyladenosine in untreated cells (Fig. 2b).

Examination of NAD and poly(ADP-ribose) levels at various times during MNNG treatment reveals a striking relationship between the decrease in NAD and the increase in poly(ADP-ribose) (Fig. 3). During the first 10 min there is a close quantitative correlation between the decrease in NAD and the

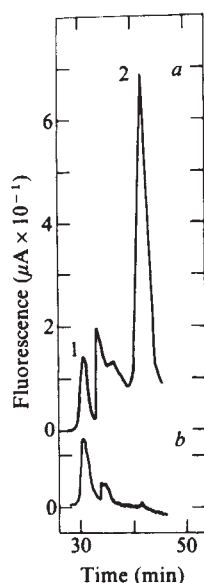


Fig. 2 Poly(ADP-ribose) determination in control and MNNG-treated SVT2 cells. Two Petri dishes of control (0 time) cells and two Petri dishes from cells treated for 20 min with 0.3 mM MNNG, containing 5.0×10^7 cells each, were subjected to the procedure outlined in Fig. 1 legend except isocratic elution was with 7 mM ammonium formate pH 5.8/100% methanol 96/4 (v/v). In each case, 50 μ l of material equivalent to 5.4×10^6 cells was injected. Chromatogram a, MNNG-treated cells; chromatogram b, control cells. The fluorometer recording range was changed to 0.1 μ A full scale at 34 min. Peak 1 is ethenadenosine and peak 2 is ethenoribosyladenosine.

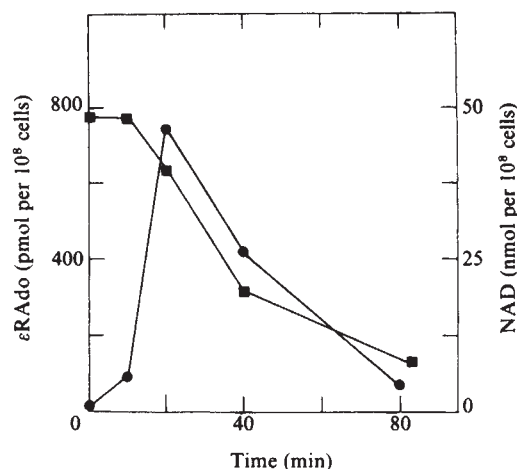


Fig. 3 Comparison of NAD and poly(ADP-ribose) content of SVT2 cells at various times of MNNG treatment. Cells from one Petri dish (5.0×10^7 cells) for NAD determinations and two Petri dishes for poly(ADP-ribose) determinations were collected at the times indicated. NAD levels represent the total of both oxidised and reduced forms and were determined as described previously²⁹. Poly(ADP-ribose) levels were determined by the method described in Fig. 1 legend using the chromatographic conditions of Fig. 2, and were corrected for overall recovery, which was 65%. For each point 50 μ l of material equivalent to 5.4×10^6 cells was injected. ■, NAD levels; ●, poly(ADP-ribose) levels. ϵ RAdo, 1,N⁶-ethenoribosyladenosine.

breaks^{25,26}. Such lesions initiate a series of reactions which lead to eventual repair of the DNA damage. The data presented here support the suggestion that one of the events leading to DNA repair is the synthesis of poly(ADP-ribose).

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accumulation of poly(ADP-ribose). However, at longer times, the rate of NAD degradation greatly exceeds the amount of poly(ADP-ribose) present, and by 20 min, when poly(ADP-ribose) is at its highest level, it can only account for 8% of the decrease of NAD. There are at least three possibilities to explain this difference. (1) The synthesis of poly(ADP-ribose) accounts for all the NAD decrease but the polymer is turning over very rapidly; (2) only a small portion of NAD is used for poly(ADP-ribose) synthesis and NAD is also being degraded by other catabolic reactions; (3) the decrease in NAD is also caused by a diminished synthesis of this nucleotide. The first explanation implies that there is an increase in the activity of both poly(ADP-ribose) polymerase and poly(ADP-ribose) degrading enzymes. The observed transient accumulation of poly(ADP-ribose) (Fig. 3) is consistent with this possibility. With regard to the other two possibilities, experiments carried out in this laboratory have shown that there is no appreciable difference in NAD glycohydrolase activity in control and MNNG-treated cells, and labelling of the NAD pool with radioactive nicotinamide has shown that NAD synthesis is not affected by MNNG (unpublished data). Thus, we favour the first possibility.

MNNG is a well known carcinogen, causing DNA alkylation which in turn results in chemical or enzymatic single-strand

An IgG primary sequence exposure theory for complement activation using synthetic peptides

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To establish the mechanism by which IgG, aggregating with antigen¹, triggers the first complement component² (the C1q·C1r·C1s complex), it is important to distinguish the potential sites on the Fc region which may be involved in the interaction. Several studies have implicated the C_H2 region of IgG³⁻⁵. C1 binds weakly to monomeric IgG but strongly to aggregated IgG^{6,7}, and an analytical ultracentrifugation study of the binding of different IgG subclasses to the C1q subunit suggested that each C1q molecule may have from 12 to 18 receptor sites for immunoglobulin⁸. Although there are conflicting reports of conformational changes in antibody molecules induced by antigens⁹⁻¹¹, electron microscopic studies of rabbit IgG antibody¹² have shown that reduction of a single disulphide bond between the heavy chains in the hinge region allows this immunoglobulin, on contact with its antigen, to undergo strain-release conformational changes in the Fc region, suggesting a potential energy release and perhaps exposure of a cryptic site. Chemical modification of the tryptophanyl residues of both rabbit and human IgG or their Fc fragments has been shown to reduce their C1-fixing potentials^{13,14}. These findings, together with examination of crystallographic structural data¹⁵ and amino acid sequence analyses^{16,17} of the C_H2 region of human IgG1, support the possibility that a tryptophanyl residue in position 277 on human IgG (near the hinge region) could be in or near a site involved in the reaction with C1q. To explore this possibility, we have synthesised a series of small peptides resembling the portion of the C_H2 region around the tryptophanyl residue at 277 (Table 1). A series of C1 consumption, C2 destruction, and C3 conversion tests was used to determine the most effective sequence for complement activation and to gain information about the functional nature of the interactions between the peptides and the human complement system. On the basis of these experiments we suggest that the primary structure of a small peptide segment is necessary for effective C1 binding and activation and that conformational changes caused by the reaction of IgG molecules with antigens, by heat aggregation, or by interfacial forces¹⁸ may expose this cryptic biologically active sequence.

Peptides were synthesised by the solution technique¹⁹, and their structures were verified by elemental and amino acid analyses. Their effects on the function of native C1 were tested by direct incubation with fresh human serum by methods previously described^{18,20}.

Peptide 98 (Lys-Ala-Asp-Trp-Tyr-Val-Asp-Gly) had the highest specific C1 consuming activity of all the peptides tested, of the same order of magnitude as heat-aggregated human γ -globulin (Table 1). Peptide 93 (Trp-Tyr-Val-Asp-Gly), identical to the sequence in IgG1 from residue 277 to 281, also had high C1-fixing activity. It is speculated that replacement of the Trp residue with a nonaromatic alanine residue (as in peptide 92) or replacement with an aromatic Tyr residue (as in peptide 88) resulted in lower complement-consuming activity.

Although few differences exist in the primary structure of this region of different IgG subclasses, antigen-complexed IgG2 is a weaker activator of C1 than is IgG1, and IgG4 is relatively inactive. In IgG2, residue 274 is Glx rather than lysine. For IgG4, again the Lys residue at position 274 is replaced by Glx, but in addition the His at 268 is replaced by Gln (ref 17). No

sequence for the same region of IgG3 has yet been reported. We postulate that the Lys 274 residue may give important conformational instruction for the active region around the tryptophanyl residue, and therefore may be responsible for the high complement-binding and activating properties of IgG1 as compared to IgG2. The very weak C1-binding and weak C1-fixing abilities of IgG4 may be due to the critical substitutions at positions 274 and 268, leading to inefficient exposure of the active complement-fixing region.

In C2 destruction experiments, each peptide was incubated for 60 min at 30 °C with a 1:200 dilution of human serum as a source of native C1 and rate-limiting C2 (ref. 21). These tests indicated that the peptides were able to activate C1 and generate C2 destruction, and thus that the major binding and activation sites on C1 may be similar or, probably, identical. Specific C2 destruction was a function of C1 consumption activity. For each peptide a minimum critical threshold concentration (CT; Table 1) was required for detectable C2 destruction. This could explain the requirement for aggregation of IgG antibody, as it indicates that a certain number of sites on C1q must bind to the peptides before C1q undergoes conformational changes and induces internal activation of C1r, leading to C1s activation. Theoretically, the relatively small synthetic peptides would be unable to cross-link the peripheral subunits of C1q; however, they might be able to generate quaternary conformational changes in C1q by altering the relative polarity of the C1q subunits, possibly through interaction with specific sites on each C1q subunit. Each C1q receptor site could conceivably contain both hydrophobic and hydrophilic receptors. When the critical number of sites become filled, we suggest that the C1q subunits could acquire a different polarity, leading to a new interaction with the polar solvent and providing favourable conditions for movement of the subunits to a position of lower free energy. These conformational changes induced by interactions with solvent might account, in part, for the subsequent internal C1 activation.

Consistent with our hypothesis are studies of the optimal ionic strength for C1 binding to antibody-coated cells^{20,22}, which suggest that charge-charge (polar) interactions may play a critical role in stabilisation of C1q binding. Likewise, C1 fixation to sensitised cells and subsequent transfer of C1 in the activated state can be accomplished by manipulation of the ionic strength of the reaction mixture²⁰.

For further confirmation that the synthetic peptides could activate the complement system, C3 conversion was monitored by crossed immunoelectrophoresis²³. Each peptide was incubated for 60 min at 37 °C with fresh human serum as a source of complement, diluted 1:4.3 in the appropriate buffer system (Table 1). Using the Ca²⁺ and Mg²⁺-containing veronal buffer system²⁰, peptides 98 (Lys-Ala-Asp-Trp-Tyr-Val-Asp-Gly), 102 (Lys-Phe-Asp-Trp-Ala-Val-Asp-Gly) and 86 (Lys-Tyr-Asp-Trp-Tyr-Val-Asp-Gly) caused conversion of C3. In the Ca²⁺ chelated controls (EGTA-Mg²⁺ buffer)²³, peptides 98 and 102 at 2.5 mg ml⁻¹ caused detectable C3 conversion whereas peptide 86 did not. This low reactivity with the alternative pathway may explain partially the absence of parallelism between the peptides' C1-activating and C3-converting activities. Note the lack of C3-converting activity by peptide 93 (Trp-Tyr-Val-Asp-Gly), which lacks the relatively polar amino acid group Lys-Ala-Asp or Lys-Phe-Asp found in the two most active peptides (Table 1).

The possible twofold role of the peptides in binding and in activating C1 is consistent with our primary sequence exposure model. We suggest that peptide 93 (Trp-Tyr-Val-Asp-Gly) probably binds the hydrophobic part of the site on C1q but may be unable to induce a net charge differential within the site. Thus, this peptide might be capable of blocking the position sought by antibody-coated indicator cells, but it would lack the charged residues and be inefficient in establishing a favourable polar environment on the subunits required for the conformational change in C1q.

Direct evidence for peptide-mediated activation of the

Table 1 Effects of synthetic peptides on the complement system

Peptide no.	Structure*	Concentration required to remove 50% C1 ($\mu\text{g ml}^{-1}$)	Concentration required for 50% C2 destruction ($\mu\text{g ml}^{-1}$)	CT† ($\mu\text{g ml}^{-1}$)	Concentration required for C3 conversion‡ ($\mu\text{g ml}^{-1}$)
98	Lys-Ala-Asp-Trp-Tyr-Val-Asp-Gly	0.07	1.80	0.50	70.00
102	Lys-Phe-Asp-Trp-Ala-Val-Asp-Gly	2.31	5.93	2.34	420.00
96	Lys-Phe-Glu-Trp-Tyr-Val-Glu-Gly-Val-Glu-Val-His-Glu-Ala-Lys-Ala-Lys-Pro-Gly-Arg	2.80	7.70	4.00	Inactive§
93	Trp-Tyr-Val-Asp-Gly	2.67	13.60	6.80	Inactive
86	Lys-Tyr-Asp-Trp-Tyr-Val-Asp-Gly	6.67	27.30	9.00	850.00
88	Tyr-Asp-Tyr-Tyr-Val-Asp-Gly	21.90	63.00	32.00	Inactive
92	Lys-Phe-Asp-Ala-Tyr-Val-Asp-Gly	33.00	61.70	45.00	Inactive
	Heat-aggregated human γ -globulin	0.05	0.13	0.05	28.00

*Sequence of IgG1 = 274 275 276 277 278 279 280 281

Lys-Phe-Asn-Trp-Tyr-Val-Asp-Gly

†CT, concentration threshold required for effects on C2 destruction.

‡Buffer system = Dextrose veronal buffered saline with Ca^{2+} and Mg^{2+} 18,20. In the EGTA- Mg^{2+} system²³, peptides 98 and 102 caused detectable C3 conversion at a concentration of 2.5 mg ml^{-1} , whereas peptide 86 did not.§No effect at 850 $\mu\text{g ml}^{-1}$.

haemolytic sequence was obtained as follows: washed sheep erythrocytes were mixed with peptide 98, 102, 96, or 93 at two different concentrations, 3.32×10^{-9} mg per cell and 56.64×10^{-9} mg per cell, in veronal buffer. The treated erythrocytes were then washed twice and added to human serum (1:6 final dilution) previously absorbed with washed but otherwise untreated sheep erythrocytes (8×10^6 cells per ml). Peptide 98 at the lower concentration (3.32×10^{-9} mg per cell) caused complement-mediated haemolysis of 50% of the erythrocytes in 21 min. Peptide 102 at the higher concentration (56.64×10^{-9} mg per cell) caused complement-mediated lysis of 50% of the cells in 28 min. None of the other peptides tested induced any measurable complement-mediated lysis in these conditions (as compared with controls). In addition, none of the peptides tested caused direct complement-mediated lysis of sheep erythrocytes in the EGTA- Mg^{2+} serum system (alternative pathway).

Assuming that the charged region of the active peptides interacted with the polar surface of the erythrocyte, the peptide's hydrophobic region would be available for binding of C1. Perhaps the proximate polar surface of the erythrocyte is able to nonspecifically substitute for or add to the charged region of the peptides and provide the required ionic interactions with C1q. This molecular situation at the membrane surface could favour the movement of the C1q subunits to a position of lower free energy, which would provide the necessary signal for internal activation of C1.

The two peptides most effective in C1 consumption, C2 destruction and C3 conversion were also the most effective in generating haemolysis of nonspecifically coated erythrocytes. It would appear, therefore, that the area of IgG required for complement-mediated haemolysis of sensitised cells could be the region with a primary sequence similar to these peptides. In addition, as indicated by the dose-response data from C2 destruction tests, it is possible that a critical saturation of the receptor sites on C1q is required for ultimate induction of C1 conformational changes leading to activation.

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Simultaneous ultrastructural demonstration of multiple peptides in endocrine cells by a novel immunocytochemical method

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Current immunocytochemical techniques detect all antibodies that react with tissue¹. Unfortunately, some of these antibodies may react with antigens other than those intended. Such problems are minimised by using sets of antibodies detecting different regions of the desired antigen^{2,3}. However, as immunocytochemical methods can now detect very low antibody concentrations¹, the purity of antisera is critical. Furthermore, although antisera may be purified by affinity chromatography, difficulties in recovering high-avidity antibodies cause most affinity-purified antisera to be enriched in low-avidity antibodies, which may be dislodged during staining¹. We have therefore developed, and describe here, a new ultrastructural post-embedding staining technique, based on the divalency of IgG molecules and using antigen-coated colloidal gold granules. Previously, colloidal gold-labelled antibodies have been used for post-embedding staining^{4,5}. Unlike our gold-labelled antigen detection (GLAD) technique, however, these methods do not differentiate between specific and nonspecific antibodies. The GLAD method detects only specific antibodies and does not select against high-avidity antibodies, and in this it resembles the radioimmunochemical method⁶. However, the GLAD method differs from the latter in that it is useful for ultrastructural studies, does not require autoradiography and allows simultaneous detection of multiple antigens. Moreover, specific activity compared with background may be quantitated.

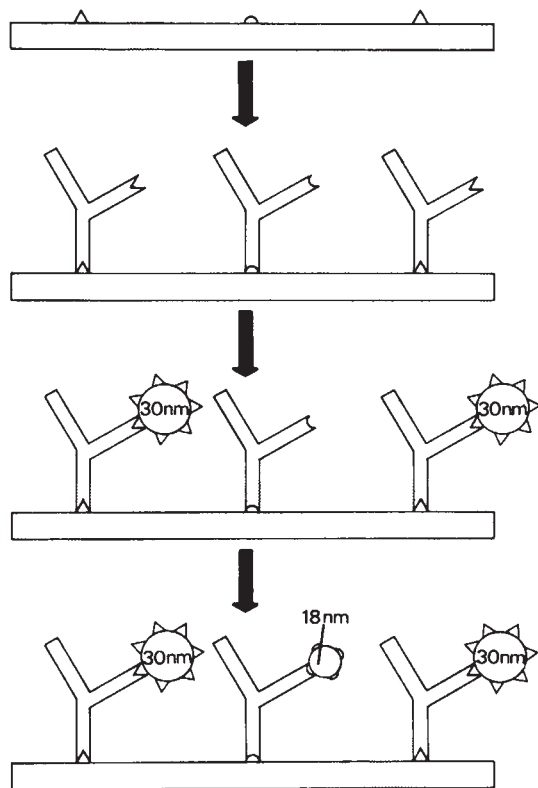


Fig. 1 The principle of the GLAD method. Bivalent IgG molecules [or $F(ab)_2$ fragments] are applied to tissue in slight excess so that they will bind with only one of their two antigen-combining sites. The remaining site is therefore free to react with subsequently added antigen-coated gold granules of defined diameters. In this example, an antiserum containing two antibody populations of different specificities has been used. With differently sized antigen-coated gold granules, the site of reaction between each of the antibody populations and their corresponding tissue-bound antigen is identified. Thus, differently sized gold granules mark sites of different antigens, whereas in conventional immunocytochemistry the two antibody populations would be confused.

Figure 1 illustrates the principle of the GLAD method. Briefly, surplus IgG molecules will react with tissue antigens with only one of their two combining sites, leaving the remaining site free to bind subsequently added antigen-coated colloidal gold granules. The gold granules will therefore indicate the site of reaction between specific antibodies and tissue antigens. Nonspecific antibodies cannot bind specific antigen on gold granules and will remain undetected. Non-immunological binding of specific antibodies to tissue is prevented by pretreatment with high concentrations of normal serum from the species delivering the antiserum.

Colloidal gold particles were prepared as described elsewhere^{7,8}. Synthetic peptides included ACTH₁₋₂₄ (CIBA), ACTH₁₋₂₈ (Ferring), β -melanocyte-stimulating hormone (β -MSH) and β -endorphin (Peninsula), caerulein (Farmitalia), human gastrin₆₋₁₃ and human gastrin₂₋₁₇ (ICI). Natural peptides were highly purified porcine ACTH₁₋₃₉ (Ferring) and human growth hormone (Nordisk Insulin). The peptides had to be conjugated to bovine serum albumin (BSA) before they could be absorbed to gold particles (details in Fig. 2 legend). Antisera were raised in guinea pigs against synthetic ACTH₁₋₂₄ (antiserum GPL 95) and porcine ACTH (antiserum GPL 71) or in rabbits to porcine ACTH₁₋₃₉ (antiserum R₁), synthetic human gastrin₁₋₁₇ (antiserum 2717, 4562), synthetic human gastrin₆₋₁₃ (antiserum 4710) or human growth hormone, coupled to bovine or human serum albumin by glutaraldehyde or carbodiimide. All antisera were therefore preabsorbed with 0.5% human or bovine serum albumin. Further controls included tests with gold granules to which only glutaraldehyde-treated BSA had been absorbed.

Pituitaries and gastric mucosa from cats and rats were used (Fig. 2). Ultra-thin sections, etched in hydrogen peroxide¹, were

treated with a 1:10 dilution of normal guinea pig or rabbit serum, exposed to various dilutions (1:20–1:20,000) of the antisera for 24 h at 4 °C, rinsed and exposed to antigen-coated gold granules (Fig. 2) in dilutions of 1:1–1:20 for 2 h at room temperature. After a brief (3 × 5 min) rinse in 0.05 M Tris-buffered saline, pH 7.4 (TBS), sections were postfixed in 1% glutaraldehyde in TBS for 30 min, briefly rinsed and contrasted with uranyl acetate and lead citrate.

Controls included: (1) omission of antisera; (2) use of antisera pretreated (24 h at 4 °C) with 1–100 μ g per ml of peptides; (3) combination of antisera with unrelated gold granules; (4) application of antibodies, followed by exposure to free antigen (100 μ g ml⁻¹, 2 h, room temperature), before exposure to antigen-coated gold granules (blocking test); (5) comparisons of results and dilutions with standard immunocytochemical methods (peroxidase–antiperoxidase (PAP) and peroxidase-labelled methods, ref. 1).

Very little nonspecific attraction occurred between gold

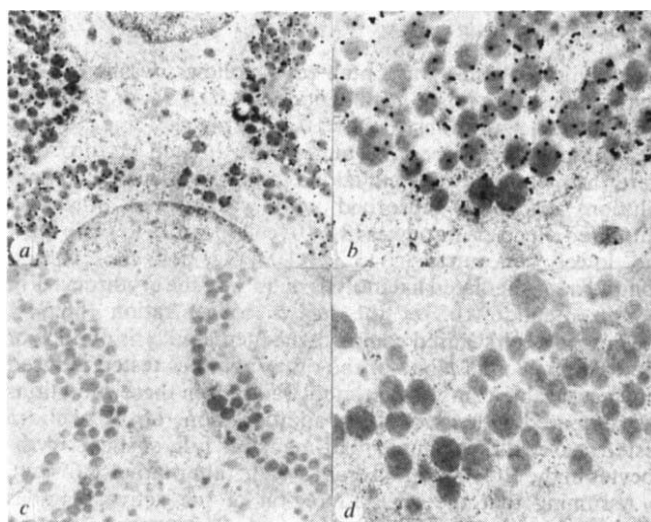


Fig. 2 Ultra-thin section of rat pituitary, stained with GH antiserum and GH-coated 18-nm gold particles (*a*: ×6,650, *b*: ×15,000). An adjacent control section was reacted with GH-inactivated antiserum and then with GH-coated 18-nm gold particles (*c*: ×6,650, *d*: ×15,000). Note the specific accumulation of gold particles over the cytoplasmic granules in *a* and *b* and the virtually absent background in the control section (*c*, *d*). Peptides used included synthetic ACTH₁₋₂₄, ACTH₁₋₂₈, β -MSH, β -endorphin, caerulein, human gastrin₆₋₁₃, human gastrin₂₋₁₇, natural porcine ACTH₁₋₃₉ and human growth hormone. Between 50 and 200 nmol peptide and 60 nmol BSA in 250 μ l 0.005 M NaCl, pH 7.0, were incubated with 50 μ l 0.25% glutaraldehyde (Merck) for 2 h at room temperature. The conjugate was diluted to 13 ml with 0.005 M NaCl and the pH adjusted to 4.0–4.5. After Millipore filtration (0.45 μ m), the conjugate was mixed with 100 ml gold granule suspension for 2 min⁸. Polyethylene glycol (molecular weight 20,000) was then added and the mixture neutralised⁸. Granules were spun down at 15,000 r.p.m. (20–30 min, 4 °C) and redispersed in 4 ml 0.05 M Tris buffer, pH 7.9, containing polyethylene glycol and sodium azide (0.5 mg ml⁻¹ of each). The granules were stable for at least 1 month at 4 °C. This procedure gives about 20–40 ml working solution of gold granules. Gold granule diameters were checked by both ultrastructural measurements and mixing experiments^{7,8}. In two experiments, 200 nmol of ACTH₁₋₃₉ and 100,000 c.p.m. ¹²⁵I-labelled ACTH₁₋₃₉ were conjugated to 60 nmol BSA. Calculation of the gold granule concentration⁸ and the recovery of radioiodinated ACTH showed that each gold granule was coated with about 70 ACTH molecules. Rats and cats were anaesthetised with ether or Mebumal, respectively, and perfused intracardially with saline, followed by a 3% formaldehyde–2% glutaraldehyde mixture in 0.1 M sodium phosphate buffer, pH 7.3, for 15 min. Specimens from pituitaries and antropyloric mucosa were postfixed for 15–30 min in the formaldehyde–glutaraldehyde mixture and routinely embedded in Epon 812 (ref. 3). Ultra-thin sections were cut on an LKB III ultratome, collected on Formvar-supported Ni grids and stained as described in the text. Chess-board titrations¹ of primary antibodies and gold granules established the concentration yielding optimal staining and lowest background. Quantitation of gold granules over adjacent sections of the same cells, stained with untreated and antigen-inactivated antisera, was carried out to quantitate background (nonspecific) staining. Quantitation of gold granules over unrelated cells gave equivalent background values. Quantitation of different subcellular compartments (cytosol compared with granules) of immunoreactive cells gave higher values than background levels, suggesting storage of peptides outside the granules.

granules and sections, being 5–7 (gold granules per μm^2 MSH cell cytoplasm; 15 areas counted) at a granule dilution of 1:2, 1–3 at 1:5 and 0–1 at 1:10–1:20. Corresponding specific staining was 120–150 at 1:2, 110–130 at 1:5 and 60–75 at 1:10–1:20 (antiserum R₁ 1:80; ACTH₁₋₃₉-coated 18-nm gold granules). Similar values were obtained with other antisera–gold granule combinations (Fig. 2). Control (1) is essential because certain peptide receptors may survive fixation and embedding¹. Such receptors may bind peptide-coated gold granules. So far, we have not detected any receptors. Antigen-inactivated (2) and unrelated antisera (3) failed to bind gold granules over background values. Blocking tests (4) showed that free antigen competed with antigen-coated gold granules for binding. Optimal dilutions of antisera ranged only 10-fold below those of the PAP method, indicating that even in conventional immunocytochemistry many antibodies do not react with both combining sites. Probably, a single IgG molecule is enough to bind a gold granule, but at sites of high antigen concentration, more antibodies may bind one granule. However, the size of the IgG molecule ($8 \times 5 \times 4$ nm; ref. 9) prevents reaction with most of the ~ 70 (Fig. 2) antigen molecules per gold granule. Note that lower amounts of antigen per gold granule (down to 10 molecules) did not produce inferior results.

The GLAD method identified gastrin-producing G cells and pituitary GH, ACTH and MSH cells. Gold granules, indicating the localisation of the hormones, were abundant over the cytoplasmic granules of these cell types (Fig. 2). Identification of cat antrotyloric G cells were carried out with antisera recognising regions 6–13 (antiserum 4710) and 14–17 (antiserum 4562) of the gastrin₁₋₁₇ molecule, respectively^{2,3}. Double staining with

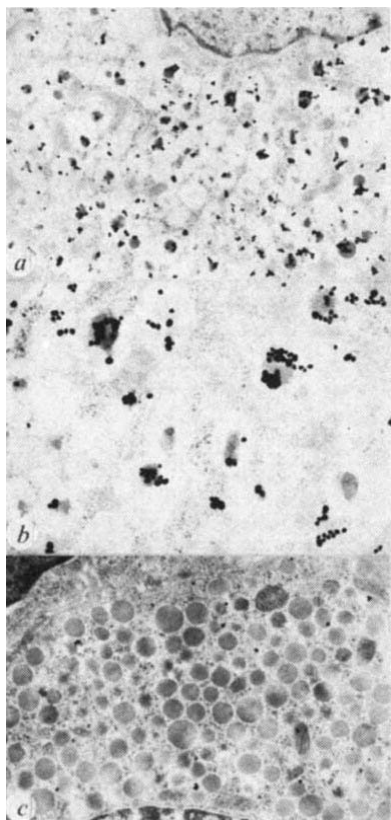


Fig. 3 Section of cat antrotyloric mucosa, stained with antisera recognising the 6–13 (antiserum 4710) and 14–17 (antiserum 4562) regions of gastrin₁₋₁₇. The site of reaction between the 14–17-specific antibodies was revealed with 18-nm gold granules coated with caerulein, a synthetic peptide containing the 14–17 region of gastrin₁₋₁₇, whereas the 6–13-specific antibodies were demonstrated with 30-nm gold granules, coated with synthetic gastrin₆₋₁₃. Note the simultaneous occurrence of both large (6–13) and small (14–17) gold granules over the dense cores of the G-cell granules. Thus, with the use of two different region-specific antibodies, the intragranular site of gastrin storage is firmly identified (a: $\times 16,900$; b: $\times 20,150$). In c, a non-gastrin-storing (D) cell in the same section is shown ($\times 8,450$). Note the low background absorption of both types of gold granule.

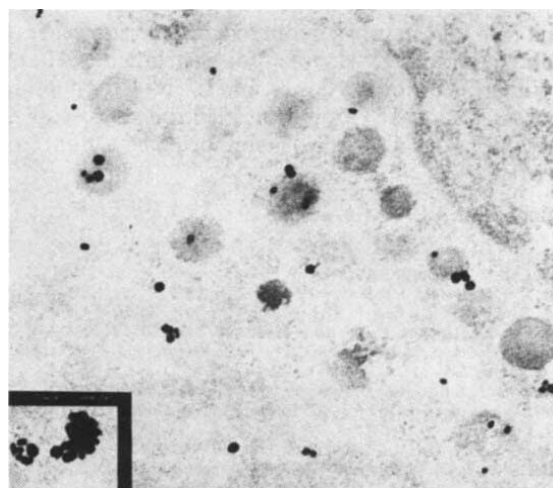


Fig. 4 Cat antrotyloric G cell, reacted with ACTH antiserum and ACTH₁₋₃₉-coated 18-nm gold particles and, subsequently, with gastrin antiserum and synthetic human gastrin₂₋₁₇-coated 30-nm gold particles. Fixation, embedding and staining are as described in the text and in Figs 2 and 3 legends. Note the occurrence of both large and small gold particles over the cytoplasmic granules, indicating their simultaneous storage of both gastrin and the ACTH-like immunoreactivity. Note also that so as not to over-shadow the few ACTH-specific gold particles, this ($\times 77,000$) shows G-cell granules, optimally stained for both gastrin and ACTH and clearly shows the masking of the 18-nm ACTH-specific gold particles by the much more numerous 30-nm gastrin-coated particles. The specificity of the staining for both ACTH and gastrin was verified as described in the text. Radioimmunochemical measurements show that the concentrations of the ACTH-like peptide in cat antrotyloric mucosal extracts averages 100 ng ACTH₁₋₃₉-equivalents per g mucosa¹⁴. These concentrations are about 100-fold lower than those of gastrin. This probably forms the background to the comparatively weaker labelling of G-cell granules by ACTH antisera. Although the ACTH-like antrotyloric peptide behaves like pituitary ACTH in two distinct gel chromatographic systems¹⁴, it cannot yet be decided whether the concentrations measured are real or are caused by poor cross-reactivity of the assay (and of the present immunocytochemical procedure) with an as yet unknown ACTH-related peptide.

these region-specific antisera, using differently sized gold granules (Fig. 3), revealed the occurrence of both regions in the electron-dense cores of the granules. Notably, G-cell granules lose their electron-dense cores following stimulation of gastrin secretion^{10,11}. Recently, we reported that cat G cells also display ACTH immunoreactivity¹². Combined use of gastrin (antiserum 4710) and ACTH (antiserum R₁) antisera, together with gastrin₂₋₁₇-coated and ACTH₁₋₃₉-coated gold granules, localised both peptides to the G-cell granules (Fig. 4). Recent studies¹⁴ have shown that the ACTH-like peptide displays radioimmunochemical and gel chromatographic properties of ACTH₁₋₃₉. In antrotyloric mucosa the concentration of this peptide is 100-fold lower than that of gastrin—an observation in agreement with the fact that many fewer ACTH-coated than gastrin-coated gold granules occurred over the G-cell granules.

One of our antisera (GPL 71), raised against crude porcine ACTH, also contained antibodies to β -MSH and β -endorphin. These peptides form, with ACTH, part of a common biosynthetic precursor¹³. Use of differently sized gold granules, coated with ACTH₁₋₃₉, synthetic β -endorphin or synthetic β -MSH, allowed the simultaneous identification of all three peptides in the secretory granules of rat MSH cells, using the 'nonspecific' antiserum GPL 71.

The GLAD method has been shown to be useful for the ultrastructural identification of various cell types and for studying multiple cellular antigens. Identification of multiple secretory peptides at the ultrastructural level allows detailed studies of hormone secretion. Several peptides have also been shown to be produced by neurones and paracrine cells. In these instances, reliable ultrastructural identification of neuronal and paracrine cell processes will help to elucidate the physiological roles of many neuroendocrine peptides. Such studies are now in progress with regard to ACTH- and enkephalin-producing nerves and preliminary results confirm the suitability of the GLAD method for this task.

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Opiate inhibition of peptide release from the neurohumoral terminals of hypothalamic neurones

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The release of peptides from the magnocellular neurones of the hypothalamus, whose axons terminate on capillaries in the neurohypophysis, has been likened to the release of neurotransmitters from conventional synapses in other parts of the nervous system¹. These oxytocin and vasopressin producing neurones generate action potentials within their perikarya, and the number and pattern of spikes propagated to the neurohypophysis govern the quantity of peptide released into the circulation^{2,3}. We do not know, however, if the release of these peptides is modified at the level of the axon terminal as occurs in conventional synapses in the form of presynaptic inhibition or facilitation^{4,5}. Within the hypothalamo-neurohypophysial system a mechanism of this type could operate either synaptically through the axo-axonal connections which are found on the neurosecretory cells⁶, or through substances reaching the neurohypophysis from adjacent tissues. The observation of enkephalin-containing fibres⁷ and opiate receptors^{2,3} within the neurohypophysis, and the presence of large amounts of β -endorphin in the adjacent parts of the pituitary gland^{8–10}, make these endogenous opioids possible candidates for such a function. Studies of lactation in mice suggest that opiates block the release of oxytocin¹¹, while a considerable body of evidence suggests that the reverse is true regarding the release of vasopressin^{12,13}. We provide here evidence that opiates inhibit the electrically induced release of oxytocin by an action on or close to the axon terminals within the neurohypophysis, and combine this with evidence to suggest that endogenous opioids tonically suppress the release of oxytocin in response to physiological stimuli.

We used lactating rats of a Wistar strain anaesthetised with urethane (1.25 g kg⁻¹, intraperitoneally i.p.). Supraoptic neurones were identified in unit recording studies by antidromic stimulation from the neurohypophysis¹⁴, and the cells were classified as oxytocinergic according to their pattern of spontaneous spike discharge and their explosive acceleration in activity some 10 s before each milk ejection induced by the sucking of the young^{2,3}. Oxytocin release was determined by

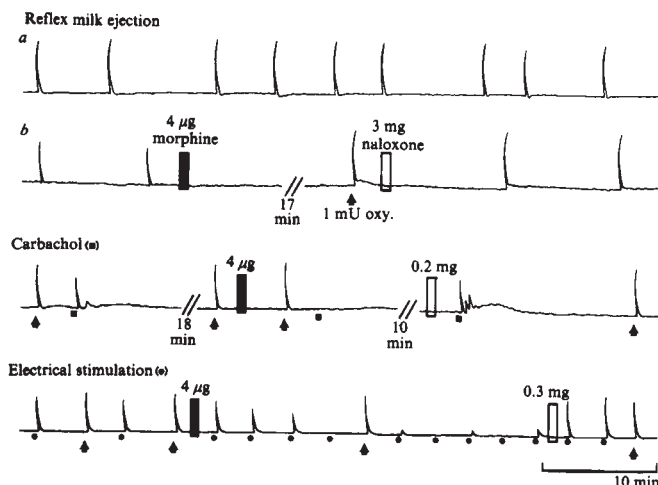


Fig. 1 Intramammary pressure recordings from four lactating rats anaesthetised with urethane to illustrate the effect of morphine on the suckling-induced and experimentally evoked release of oxytocin. Reflex milk ejection was induced by continuous sucking of the uncannulated nipples by a litter of 10 pups. *a*, Normal pattern of milk ejection, characterised by uniform rises in intramammary pressure at intervals of 5–10 min. *b*, This intermittent pattern of oxytocin release has been abolished by i.v.t. injection of morphine (4 µg). Carbachol (■), 0.2 µg injected into the cerebral ventricles, evoked a mammary contraction of several minutes' duration; no evidence of oxytocin release was observed in response to this pharmacological treatment after administration of morphine. Similarly, the amount of oxytocin released by electrical stimulation of the neurohypophysis (●) (5 s at 50 Hz) was reduced progressively and finally eliminated by i.v.t. injection of morphine. Naloxone totally reversed all three examples of morphine-induced inhibition of oxytocin release. Neither morphine nor naloxone changed the sensitivity of the mammary glands to exogenous oxytocin; the arrows each indicate a bolus injection of 1 mU oxytocin into a saphenous vein.

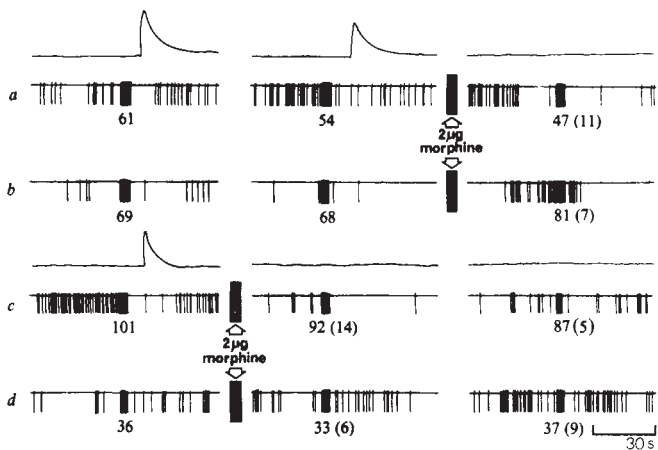


Fig. 2 Recordings from four antidromically identified oxytocinergic neurones taken from the supraoptic nucleus of four rats anaesthetised with urethane: these illustrate the failure of morphine to modify the patterns of electrical activity induced by the sucking of the pups. Each deflection of the unit recording traces (*a–d*) corresponds to a single action potential. Above recordings (*a–d*) are shown the simultaneous recordings of intramammary pressure. These recordings of reflex milk ejection differ from those illustrated in Figs 1 and 3 due to the operation of the chart-recorder at a faster speed. Three periods of electrical activity are shown for each oxytocinergic cell; these were selected to span three consecutive bursts of high frequency discharge. Before i.v.t. injection of morphine these bursts of activity were followed by milk ejection after a latency of about 10 s, as indicated in this figure by the increase in intramammary pressure. The number of action potentials in each neurosecretory burst is given, and after administration of morphine the interval from the previous neurosecretory burst is stated in minutes in parentheses. The neurosecretory bursts observed after morphine treatment closely resembled those seen earlier, although there were no rises in intramammary pressure.

measurement of intramammary pressure. Three forms of stimulation were applied: (1) sucking of 10 hungry pups at 8 d of age; (2) intraventricular (i.v.t.) injection of carbachol (0.2 μ g in 1 μ l), and (3) electrical stimulation of the neurohypophysis (4–5 s at 50 Hz, biphasic 1-ms square waves). Morphine and opiate-like peptides were administered by i.v.t. injection, while the opiate antagonist naloxone was given intravenously (i.v.).

Three hours after the onset of anaesthesia the pups were applied to the nipples of the doe and allowed to suck continuously for several hours. Individual milk ejections were observed at intervals of 5–15 min (Fig. 1): these followed the pattern described previously¹⁵. This intermittent pattern of oxytocin release was eliminated for a significant period in 11/12 rats after administration of 2–4 μ g morphine sulphate (i.v.t.), and the reflex was restored within 3–18 min in 6/7 rats by treatment with naloxone (1 or 10 mg kg⁻¹) (Fig. 1). Milk ejection was inhibited for >60 min in those rats not given naloxone.

Twenty-three oxytocinergic neurones were studied in a further group of rats: all displayed an explosive 2–4-s burst of spike activity about 10 s before each milk ejection (Fig. 2). Six cells were studied for more than an hour so that the effects of both morphine and naloxone could be evaluated. In all six cells and in others in which partial recordings were obtained, morphine (2 μ g, i.v.t.) failed to modify the pattern of electrical activity and bursts of spike activity recurred with the same periodicity and magnitude observed previously, though oxytocin was not released (Fig. 2). After administration of naloxone (1 mg kg⁻¹, i.v.) these bursts of spike activity were once again associated with oxytocin release.

The i.v.t. injection of carbachol evoked a sustained release of oxytocin as judged from recordings of unit activity and

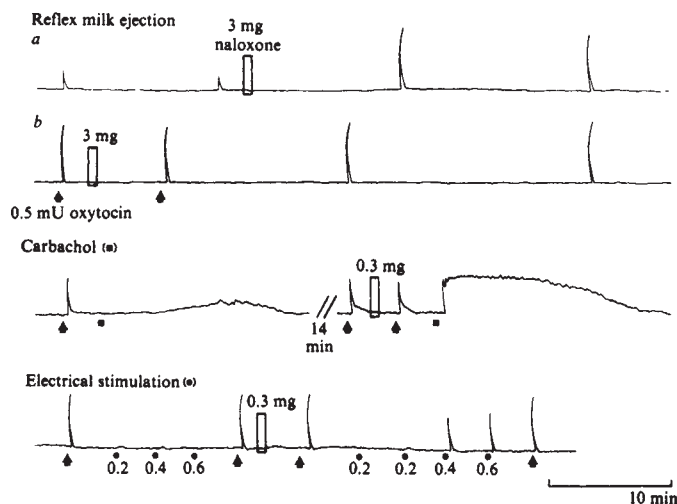


Fig. 3 Intramammary pressure recordings from four lactating rats to illustrate that naloxone increases the amount of oxytocin released in response to physiological and experimental stimuli. None of the animals was pretreated with morphine or given any opioid peptide. Reflex milk ejection was evoked by the sucking of the uncannulated nipples of the mother by a litter of 10 pups. Trace *a* commences with the fifth milk ejection after the application of the pups to the nipples. In recording *b* no milk ejections were observed in the previous 60 min of observation. The administration of naloxone (3 mg kg⁻¹, i.v.) increased the amplitude of the mammary contractions observed in recording *a*, and promoted the onset of contractions in recording *b*. Similarly, naloxone (0.3 mg kg⁻¹, i.v.) markedly increased the amount of oxytocin released by i.v.t. injection of carbachol (0.2 μ g, ■). The last recording illustrates several attempts to induce release of oxytocin by electrical stimulation of the neurohypophysis (●) (4 s at 50 Hz). The values given below each period of stimulation represent the estimated position of the electrode tip (mm) below the surface of the neurohypophysis. Before naloxone treatment stimulation at depths of 0.4 and 0.6 mm was ineffective. On no occasion did naloxone increase the sensitivity of the mammary glands to exogenous oxytocin. Within each recording illustrated the injections of oxytocin (arrows) remained constant throughout.

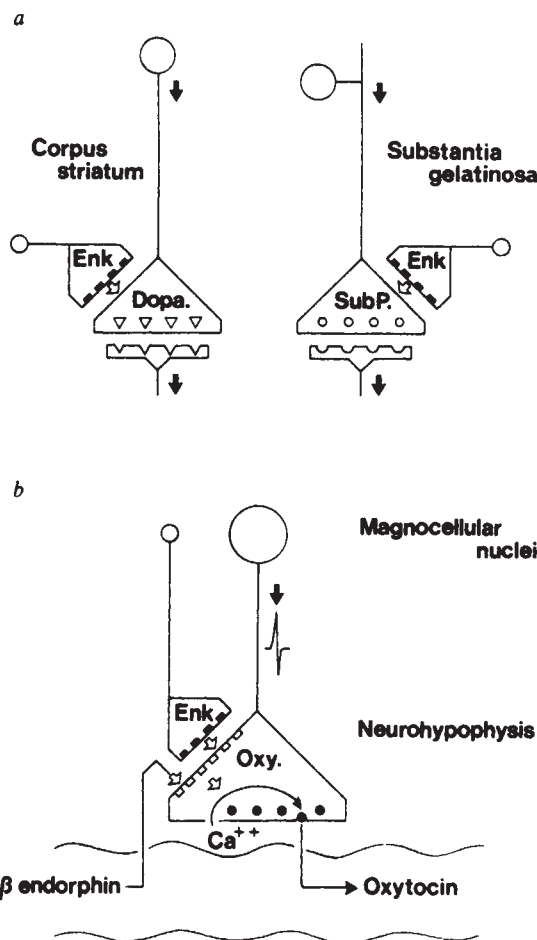


Fig. 4 Illustration to represent the similarities between the postulated roles of opioid peptides in the regulation of three different parts of the nervous system. The diagram follows the format used by Jessell and Iversen¹⁸. *a* Depicts published data in which it has been shown that opiates appear to block neural transmission in the corpus striatum and spinal cord by a presynaptic inhibitory action on dopamine- and substance P-containing terminals, respectively. *b* Illustrates by the same design, the opiate inhibition of oxytocin release observed in the present study. In this neurohumoral system β -endorphin of pituitary origin could be the effective agent, and this might reach the neurohypophysis through the vascular circulation as shown. Enk, enkephalins; Dopa, dopamine; SubP, substance P; Oxy, oxytocin.

intramammary pressure¹⁶ (Fig. 1). This release was reduced by pretreatment with morphine (4 μ g, i.v.t.) in 5/6 rats, and restored by naloxone (0.6 mg kg⁻¹, i.v.). Electrical stimulation of the neurohypophysis at intervals of 3–5 min elicited a uniform rise in intramammary pressure equivalent to that evoked by a bolus injection of about 1 mU oxytocin, i.v. The amount of oxytocin released by electrical stimulation of the neurohypophysis was substantially reduced in 5/5 rats within 6–12 min of administration of 2–4 μ g morphine, and abolished in a further three animals. The response to electrical stimulation was restored immediately by naloxone (1 mg kg⁻¹, i.v.).

With none of these treatments was the intramammary pressure response to the i.v. injection of oxytocin either attenuated by morphine (200 μ g, i.v.t. or 5 mg kg⁻¹, i.v.) or potentiated by naloxone (10 mg kg⁻¹, i.v.). These studies therefore indicate that morphine, while not changing the electrical activity generated by the oxytocinergic cells, reduces the amount of peptide released, and the action must be on or close to the terminals within the neurohypophysis. Furthermore, the occurrence of identical bursts of action potential activity after treatment with morphine and naloxone indicates that no opioid

synapse is involved directly in the transmission of the sucking stimulus from the nipples to the hypothalamus.

Evidence for an inhibition of oxytocin release by endogenous opioids was obtained by examining the effects of naloxone in the absence of morphine. In 9/9 rats the intramammary pressure responses elicited by the sucking of the pups were increased after administration of naloxone (10 mg kg^{-1} , i.v.), although the periodicity from one milk ejection to the next remained unaltered (Fig. 3), and in two rats which had previously failed to eject milk, pressure responses appeared after naloxone. Similarly in 6/9 rats electrical stimulation was more effective after naloxone, and the same treatment potentiated the amount of oxytocin released by carbachol in 7/12 rats. These observations can be interpreted in two ways. First, urethane could have a sustained opiate-like action which is reversed by naloxone. A more attractive possibility is that endogenous opioids tonically suppress the release of oxytocin. Some support for this possibility is provided by pilot studies in which a similar inhibition of oxytocin release has been observed after treatment with β -endorphin and (D-Ala, Met)-enkephalin.

Our observations have several parallels with results obtained for other parts of the nervous system. Opiates seem to mediate presynaptic inhibition on the small-diameter sensory fibres entering the spinal cord¹⁷ and brain stem¹⁸, and on dopaminergic neurones in the striatum¹⁹, and it has been suggested that this inhibition involves the mechanism of calcium uptake by the terminals²⁰. Some parallels between these different systems are illustrated in Fig. 4. If, as these studies suggest, opiates have a common mode of action within such diverse systems, then the dissociation of electrical activity from peptide release observed in our study could well apply to more closely related neuroendocrine mechanisms. Opiates have been shown to cause a release of prolactin and growth hormone from the adenohypophysis²¹ and to suppress the release of luteinising hormone (LH) and thyroid-stimulating hormone (TSH)²², while naloxone has in some cases the opposite action. The release of prolactin and growth hormone is largely restrained by dopamine and somatostatin of hypothalamic origin, while the release of LH and TSH depends on the output of the appropriate hypothalamic releasing factors, gonadotropin- and thyrotropin-releasing hormones. If opiates restricted the output of dopamine and these other hypothalamic peptides then one would expect the plasma levels of prolactin and growth hormone to rise, while those of LH and TSH would fall or remain low. More significantly, such changes in pituitary function would be largely independent of the electrical activity of the hypothalamic neurones synthesising these factors.

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Regulation of glutamate receptors by cations

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Current evidence suggests that glutamate is a major excitatory neurotransmitter in the mammalian central nervous system (CNS)¹; particularly, glutamate excites most neurones in the CNS. Until recently this effect was widely used to study glutamate receptors and to distinguish them from those of other excitatory amino acids². The development of ligand binding studies for many neurotransmitters has facilitated the study of receptors at the molecular level and using these methods we recently reported the existence in hippocampal membranes of pharmacologically distinct sodium-dependent and sodium-independent glutamate binding sites³, the former related to high-affinity uptake and the latter exhibiting several characteristics of postsynaptic receptor sites⁴. We now report that, as with other neurotransmitters^{5–8}, several ions regulate the Na-independent binding of glutamate; the monovalent cations induce a decreased binding while certain divalent cations enhance this Na-independent binding. Additionally, since some of these effects appear to be irreversible, we propose that the regulation of glutamate binding by cations might account for the extremely long-lasting potentiation of synaptic responses found in the hippocampus following bursts of repetitive electrical stimulation (see ref. 9 for a review).

Several cations were tested for their ability to modify the specific Na-independent glutamate binding to hippocampal membranes, assayed at a ³H-glutamate concentration of 100 nM. The monovalent cations, sodium, lithium, caesium, rubidium and potassium all markedly inhibited this binding in a dose-dependent manner (Fig. 1). Sodium was the most potent cation with a significant inhibition at a concentration as low as 0.5 mM; however, the maximal degree of inhibition was difficult to assess as at concentrations above 10 mM, sodium stimulates glutamate binding, thus revealing the Na-dependent binding related to high-affinity uptake sites³. Lithium was also very potent in inhibiting glutamate binding, with 50% inhibitory concentration (IC₅₀) of about 6.3 mM and maximal inhibition of about 90%. Caesium and rubidium were equally effective, exhibiting an IC₅₀ of about 20 mM. Potassium was the least potent with an IC₅₀ of about 55 mM. This effect was not related to the chloride anion associated with the cations since bicarbonate salts produced similar effects. None of these cations modified the nonspecific glutamate binding, which was determined in the presence of 100 μ M of cold glutamate and which represented less than 10% of the total binding, at the concentration of ³H-glutamate used in this assay. To determine whether the observed inhibition was due to a change in the affinity or in the number of glutamate receptors, the binding of various concentrations of ³H-glutamate was measured at two different lithium concentrations (2.5 mM and 10 mM) and the results analysed by the method of Scatchard (Fig. 2). Lithium chloride decreased the number of ³H-glutamate binding sites without affecting the apparent affinity of glutamate for the receptors.

Several divalent cations were also tested for their ability to modify the specific Na-independent binding in the same conditions. Calcium and manganese were found to be very potent in enhancing ³H-glutamate binding, producing significant increases at concentrations as low as 25 μ M (Fig. 1). The effect of both cations was maximal at a concentration of about 1 mM, at which they increased binding by about 100%, whereas concentrations higher than 5 mM resulted in an inhibition of

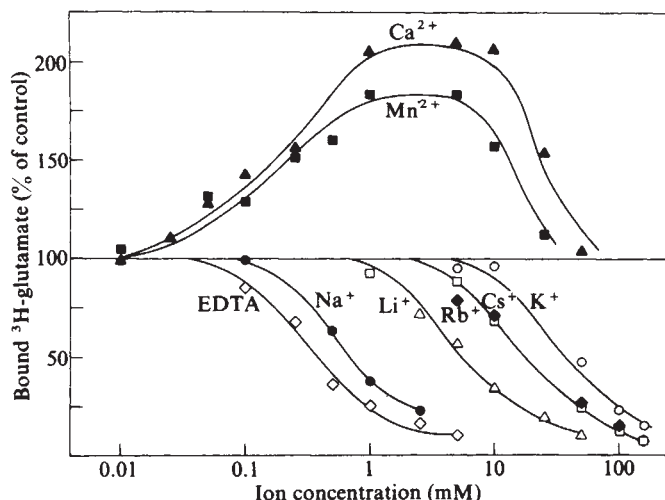


Fig. 1 Effects of various cations on ^3H -glutamate binding. Sprague-Dawley rats (150–200 g) were decapitated, the hippocampus quickly dissected, and homogenised in 40 vol. of 0.32 M sucrose using a glass-Teflon homogeniser. Membranes were prepared according to the procedure of Enna and Snyder¹⁰, except that the final pellet was resuspended by sonication in 50 mM Tris-HCl buffer pH 7.4 (about 0.5–1.0 mg protein ml^{-1}). ^3H -glutamate binding was assayed as previously described³. After 10 min preincubation at 30°C, 100 nM ^3H -glutamate (46 Ci mmol^{-1} , NEN) was added and the incubation continued for 15 min. The incubation was stopped by adding cold Tris-HCl buffer and filtration on Millipore cellulose filter (0.45 μm pore size). Filters were washed twice with 4 ml of cold buffer, extracted with 3.5 ml of antineutrotic cytotoxic serum (Amersham) and counted in a Beckman scintillation counter with 40% efficiency. The cations were added in the preincubation and in most of the cases as chloride salts. Similar results were obtained with the bicarbonate salts of sodium, rubidium and caesium. Results are expressed as percentage of specific binding (determined as the difference between total and binding in the presence of 100 μM cold glutamate) in the absence of added cation. Each point is the mean of two or three experiments which differed by less than 5%.

^3H -glutamate binding. Magnesium also increased ^3H -glutamate binding by about 20% at a concentration of 5 mM, whereas cobalt and barium were ineffective at up to 10 mM and inhibited ^3H -glutamate binding at higher concentrations. Scatchard analysis of ^3H -glutamate binding in the presence of 1 mM calcium revealed that calcium increased the maximum number of sites without changing the apparent affinity of glutamate for the receptors (Fig. 2). In addition, calcium (1 mM) was able to antagonise the decreased binding elicited by lithium chloride (2.5 mM, data not shown). The selective effects of low concentrations of some divalent cations on ^3H -glutamate binding suggested that endogenous cations present in the membrane preparation could regulate ^3H -glutamate binding. Therefore, we tested whether treatment of membranes with chelating agents could result in changes in the binding of ^3H -glutamate opposite to those elicited by the divalent cations. EDTA produced a marked and dose-dependent reduction in ^3H -glutamate binding, inducing a 90% inhibition of the binding at a concentration of 5 mM (Fig. 1). EGTA was also able to decrease ^3H -glutamate binding but to a lesser extent than EDTA (25% reduction at 0.5 mM EGTA, data not shown). As in the previous cases, Scatchard analysis of ^3H -glutamate binding in the presence of 0.25 mM EDTA indicated that the chelating agent was decreasing the number of glutamate binding sites without changing the apparent affinity for glutamate (Fig. 2). These results suggested that an endogenous divalent cation present in the membrane preparation was regulating glutamate binding. In fact, calcium, magnesium and manganese, but not nickel or copper, were able to counteract in a dose-dependent manner the

inhibition of ^3H -glutamate binding elicited by 0.25 mM EDTA (data not shown).

To determine whether the above described effects were reversible, we pretreated the membranes with EDTA or calcium and then measured glutamate binding after washing to eliminate these agents (Fig. 3). Hippocampal membranes pretreated with EDTA still exhibited a decreased binding, while membranes pretreated with calcium showed an increased binding as compared with untreated membranes, although these changes were smaller than those found when the binding was measured in the presence of these agents (Fig. 3).

Recently, Forster and Roberts¹¹ described a high-affinity ^3H -glutamate binding site in cerebellar membranes which exhibited characteristics expected of a postsynaptic receptor. Using hippocampal membranes, we found a similar homogeneous population of high-affinity ^3H -glutamate binding sites assayed in the absence of sodium. Another population was revealed in the presence of 150 mM sodium and is probably related to high-affinity uptake sites (ref. 3, and Baudry and Lynch, in preparation). In the present study we showed that the so-called Na-independent binding sites are markedly affected by divalent cations. Whereas the monovalent cations induced an inhibition of ^3H -glutamate binding, some divalent cations increased the binding. Since similar effects of ions have already been reported for several neurotransmitter, opiate and benzodiazepine receptors^{5–8,12–14}, these results strengthen the hypothesis that ^3H -glutamate labels postsynaptic receptors in these conditions.

There are several types of mechanisms which might be responsible for the extreme lability and cation sensitivity of the glutamate binding sites. Neurophysiological studies have suggested that the glutamate receptor is tied to sodium conductance channels¹⁵ and by analogy with other systems (for example, γ -aminobutyric acid and chloride⁷, glycine and chloride⁵, benzodiazepine and chloride¹⁴) it is possible that the Na⁺-induced decrease in binding is due to some interaction between the sodium channel and the glutamate receptors. On the other hand, calcium is known to have potent effects on membrane organisation including junctions between cells¹⁶ or

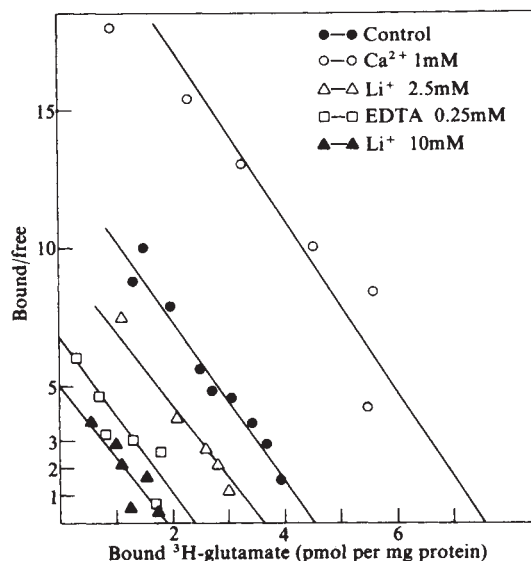


Fig. 2 Scatchard analysis of ^3H -glutamate binding in the presence of various cations. Assay conditions as described in Fig. 1, with a fixed amount of ^3H -glutamate (100 nM) and varying concentrations of unlabelled glutamate. The characteristics of ^3H -glutamate binding (K_d and B_{max}) were determined by linear regression analysis and were found to be: controls (●), $K_d = 660$ nM, $B_{\text{max}} = 4.5$ pmol per mg protein; calcium (○), $K_d = 790$ nM, $B_{\text{max}} = 8.2$ pmol per mg protein; lithium 2.5 mM (△), $K_d = 620$ nM, $B_{\text{max}} = 3.6$ pmol per mg protein; EDTA (□), $K_d = 750$ nM, $B_{\text{max}} = 2.4$ pmol per mg protein; lithium 10 mM (▲), $K_d = 750$ nM, $B_{\text{max}} = 1.9$ pmol per mg protein. Mean of two to three experiments.

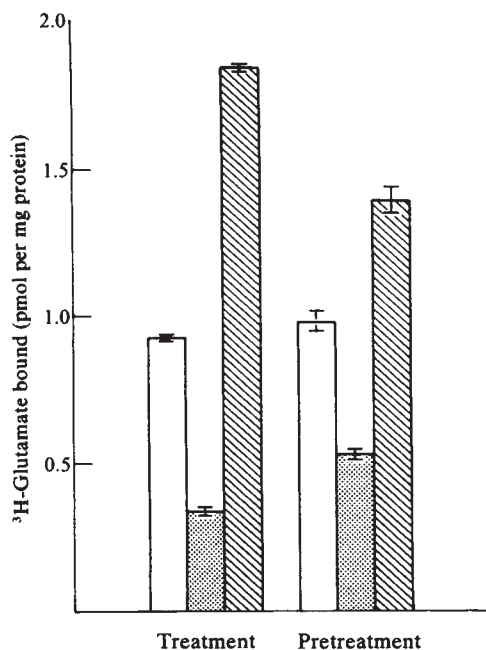


Fig. 3 Effects of pretreatment with EDTA or Ca^{2+} on ^3H -glutamate binding. Hippocampal membranes were prepared as described in Fig. 1. Aliquots (1 ml) were preincubated for 10 min in the absence (controls; open bars) or presence of EDTA (1 mM, stippled bars) or Ca^{2+} (1 mM, cross-hatched bars). After a 10-fold dilution, they were centrifuged at 40,000g for 30 min and the pellet reconstituted in 1 ml of Tris-HCl buffer pH 7.4 by sonication. This procedure therefore resulted in an approximately 100-fold dilution of the initial concentration of EDTA or Ca^{2+} . Binding of ^3H -glutamate (100 nM) was assayed as in Fig. 1. Mean $\pm$ s.e.m. of three determinations and the experiment was repeated three times. All the changes were statistically significant at $P < 0.005$.

stimulating neurofilament disruption^{17,18} and increases in receptors produced by this ion might reflect an action of this type (especially if calcium was acting on the intracellular side of the isolated membrane). Alternatively, it might be that glutamate receptors exist in two configurations, as it has been suggested for several neurotransmitter receptors¹⁹.

While the physiological significance of the above results is unclear, the lability of glutamate receptor numbers combined with the partial irreversibility of the calcium effect provides a possible explanation for the extraordinarily persistent potentiation of synaptic transmission found in hippocampus after brief periods of repetitive stimulation⁹. Several lines of evidence suggest that glutamate or one of its analogues is the transmitter in the synapses in question²⁰ and an increase in the glutamate receptor number could certainly produce an enhanced synaptic response. (It is noteworthy that the induction of the long-term potentiation is quite sensitive to extracellular concentrations of calcium²¹.) In preliminary studies using *in vitro* slices of hippocampus we have found that the potentiation effect is correlated with an enduring (at least 30 min) increase in the accumulation of a low concentration of ^3H -glutamate in slices²².

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Mutant avian erythroblastosis virus with restricted target cell specificity

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Avian erythroblastosis virus (AEV) induces a fatal erythroblastosis within 2 weeks of intravenous injection in chicks in virtually 100% of cases. In chicks injected intramuscularly, sarcomas frequently develop at the site of injection before the animals die from erythroblastosis¹. *In vitro*, AEV transforms both erythroblasts, derived from bone marrow cultures, and fibroblasts. These effects have been shown to be a general property of AEV and not of separate leukaemia- and sarcoma-inducing forms of the virus². AEV is defective for replication and can be propagated only in the presence of helper virus. Its transformation specificity is independent of the helper virus used². It is not clear whether AEV has two different genes controlling transformation of the two types of target cell or whether it has only one gene coding for both. To investigate this question, we looked for mutants of AEV unable to transform one of the two types of target cell. We now describe such a mutant, which is defective for erythroblast transformation but which can still transform fibroblasts.

To mutagenise the virus, chicken embryo cell cultures (consisting mainly of fibroblasts) transformed with AEV-ES4 (ES4AV)² were treated with 5-azacytidine (25 $\mu\text{g ml}^{-1}$, Calbiochem) overnight at 35 °C. The supernatant was frozen at -70 °C as mutagenised virus stock. Mutants were isolated as follows: 3×10^6 fibroblasts per 100-mm dish were infected with different dilutions of mutagenised AEV. One day later cells were trypsinised and seeded in semi-solid medium containing 1.5 % methylcellulose (Methocel) in 50-mm dishes at 1×10^6 cells per dish². After 10-14 d of growth at 35 °C, transformed colonies were isolated as before² and seeded into the wells (15 mm) of multiple-well tissue culture trays containing ES4AV. This helper virus² was added to ensure that every isolated clone produced infectious virus since the chance of isolating non-producer clones in these conditions was rather high². After growth to confluency, the supernatant of each clone was passaged once more through fresh fibroblast cultures at

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35 °C and then stored at -70 °C before analysis. Because our initial goal was to isolate temperature-sensitive (ts) mutants, each virus clone was tested for transformation of fibroblasts at 35 °C and 41 °C in the focus assay². Most clones were also tested for transformation of bone marrow cells at 35 °C and 39 °C. From about 500 clones screened, about 30 % were discarded because they produced no or little transforming virus, and about 10% candidate mutant clones were lost on retesting. No stable mutant temperature-sensitive for fibroblast or erythroblast transformation could be obtained. Instead, one mutant completely unable to transform erythroblasts in culture was found. This mutant is designated *td359* AEV, *td* referring to transformation defective.

Table 1 shows the capacity of *td359* AEV to transform fibroblasts and bone marrow cells in comparison with a stock of wild type (*wt*) AEV (ES4AV). The mutant induced no transformation of bone marrow cells although it had an even higher fibroblast-transforming titre than the parental AEV. As judged by the plaque assay³, both viruses contained an excess of helper viruses in the stocks used.

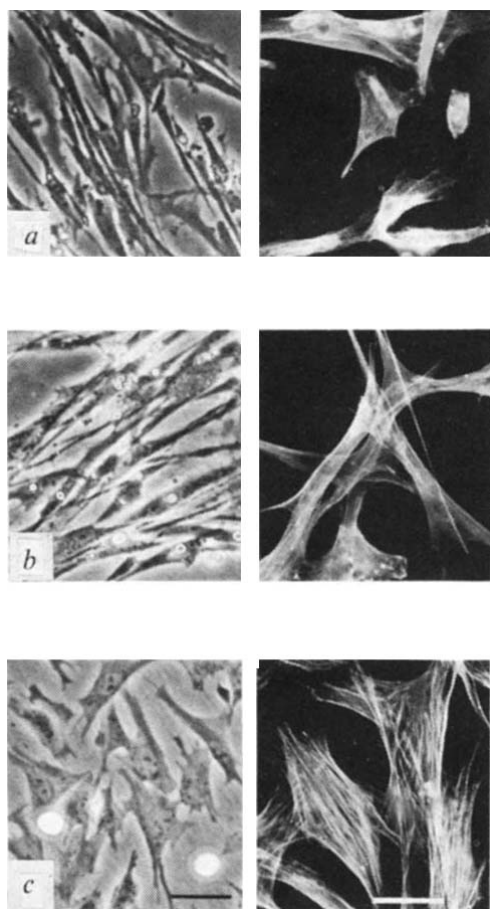


Fig. 1 Comparison of *td359* AEV- and *wt* AEV-transformed chicken fibroblasts. Left panel: phase micrographs; right panel: fluorescence micrographs of cells stained with anti-actin serum⁸. *a*, *td359* AEV clone 1; *b*, *wt* AEV clone 2; *c*, tertiary chicken embryo fibroblasts. Bars correspond to 50 μ m.

To determine whether fibroblasts transformed by *td359* AEV differ from those transformed by *wt* AEV, clones transformed by one or the other virus were compared for the expression of various transformation parameters. As Fig. 1 shows, fibroblasts transformed by *td359* AEV were essentially indistinguishable in

morphology and staining of actin cables from fibroblasts transformed by *wt* AEV but they differed from fibroblasts transformed by Rous sarcoma virus (RSV)⁸. Likewise, foci and colonies in Methocel, induced by *td359* AEV, were identical to those induced by *wt* AEV and different from those induced by RSV². Quantitative data on the expression of these parameters, of the capacity of *td359* AEV to induce casein plaques (a measure of the plasminogen-activator activity of the infected cells), and of the hexose uptake rate of *td359*-AEV-infected fibroblasts are shown in Table 2. Thus the mutation in *td359* AEV does not detectably affect the ability of AEV to transform fibroblasts as measured by the expression of these transformation parameters.

Table 1 Assay of *td359* AEV in fibroblast and bone marrow culture

	Virus assayed in		
	fibroblasts (FFU per ml)	bone marrow (PFU per ml)	bone marrow (CFU per ml)
<i>td359</i> AEV (ES4AV)	5.8×10^4	4.0×10^6	0
<i>wt</i> AEV (RAV-2)	2.5×10^4	2.0×10^6	7.5×10^2

FFU, focus-forming units; PFU, plaque-forming units; CFU, colony-forming units.

These properties of the mutant were stable even after several passages in fibroblast cultures. In addition, the virus could be propagated in bone marrow macrophages (which remained untransformed) without affecting its properties (our unpublished observations).

To demonstrate that the inability of *td359* AEV to transform erythroblasts was not due to a mutation in the helper virus, we sought to isolate clones of fibroblasts infected with the mutant in the absence of helper virus. Primary or secondary fibroblast cultures were infected with various dilutions of *td359* AEV (ES4AV), seeded in medium containing Methocel, and 12 d later colonies of transformed cells were isolated from dishes containing less than about 20 colonies. Several clones were then grown up and their supernatants were tested for reverse transcriptase activity as before⁴. In a first experiment, one out of five clones proved negative; in a second experiment six out of six were negative. To rescue transforming virus, parallel cultures obtained from one of these clones were superinfected with 10^4 – 10^5 infectious units of standard helper viruses of subgroups A to D and the supernatants were scored in focus and bone marrow-colony assays 6 d later. Table 3 shows that fibroblast-transforming virus could be rescued with all of the helper viruses

Table 2 Expression of transformation parameters in *td359*AEV-infected chicken embryo fibroblast cultures

	Focus test ² (FFU per ml)	Type of assay (titre) Colony assay ² (CFU per ml)	Casein test ⁸ (PFU per ml)	Cells with actin cables ^{8*} (%)	2-Deoxy- glucose uptake ^{8*} (c.p.m. per 10 ⁶ cells)
<i>td359</i> AEV	3 × 10 ³	4.8 × 10 ³	4.1 × 10 ⁴	23	125,000
<i>wt</i> AEV	9 × 10 ³	1.0 × 10 ⁴	1.0 × 10 ⁵	26	145,000
Uninfected control	<10 ¹	<10 ¹	<10 ¹	92	27,400

* Average of two determinations. Measurements on *td359* AEV- and *wt* AEV-transformed cells were performed on two fibroblast clones derived from colonies isolated from Methocel-containing cultures. All cells were grown at 41 °C.

and that, as with the original stock of the mutant, none of the *td359* AEV pseudotypes could induce transformation of bone marrow cells. As a control, in the same experiment, bone marrow cells were infected with a 10^{-1} dilution of *wt* AEV (RAV-2). In the infected culture, 60 transformed colonies were

obtained. In bone marrow cultures infected with undiluted *td359* AEV (RAV-2), about 30 colonies and in those infected with *td359* AEV (*tdSR-H*), about 50 colonies, were observed which were similar but larger and more compact than the normal macrophage-granulocyte colonies seen with uninfected bone marrow cells^{5,6} or when infected with the respective helper viruses only. Repeated attempts to clone and cultivate these colonies failed, in contrast to consistent results with *wt* AEV-induced erythroblast colonies.

To determine whether *td359* AEV is also unable to induce erythroblastosis *in vivo* and whether it retained the ability of *wt* AEV to induce sarcomas, two types of experiments were performed. First, 10 chicks (10-d-old) were injected intravenously in the left wing with about 1×10^4 FFU of *td359* AEV (RAV-1). A pseudotype with the helper virus RAV-1 was chosen because this virus had been shown to induce no or little anaemia². As a control, five chicks were injected similarly with *wt* AEV (RAV-1). All animals were then screened twice weekly for 3.5 months for erythroblastosis (by preparing stained blood smears) and for sarcomas (by manual palpation). As Table 4 shows, in contrast to results obtained with *wt* AEV, no animals given *td359* AEV died of erythroblastosis. One animal, however, showed a transient appearance of a few blast-like cells in the peripheral blood 45 d after injection, and in another case a solid tumour developed at the site of injection, being first observed 55 d after injection, and reaching about the size of a peanut by the end of the experiment. The low incidence of tumour formation could have been a consequence of the mode of injection: even with *wt* AEV, few sarcomas are obtained after intravenous injection¹.

Table 3 *In vitro* transformation host range of *td359* AEV pseudotypes obtained after superinfection of nonproducer fibroblasts

Helper virus used for superinfection	Virus assayed in		
	(FFU per ml)	(PFU per ml)	(CFU per ml)
None	0	0	0
RAV-1 (A)	4.5×10^4	0	0
RAV-2 (B)	2.0×10^4	8.7×10^5	0
<i>tdB77</i> (C)	1.5×10^4	0	0
<i>tdSR-H</i> (D)	2.0×10^4	5.5×10^5	0

To verify that *td359* AEV is indeed sarcomagenic a second experiment was performed in conditions known to yield a maximum number of sarcomas with *wt* AEV¹. Chicken embryo fibroblasts were infected with *td359* AEV (RAV-1) and passaged twice until all cells appeared to be morphologically transformed. These cultures were then trypsinised and inoculated into wing webs of six 5-d-old chicks at various doses (1×10^6 , 2×10^6 and 5×10^6 cells into each of two animals). Again, the chicks were monitored at 3–4-d intervals for erythroblastosis and sarcomas for 70 d. The results summarised in Table 4 show that all chicks developed tumours at the site of injection. They were detected between 13 and 28 d after injection and in one chick the tumour regressed about 50 d after injection. No animals developed leukaemia, although there was an increase in circulating monocytes, granulocytes and thrombocytes in some of those with tumours. At the time of death the largest tumour weighed 42 g (total body weight 373 g) and the others varied between the size of a pea and a walnut. Histological examination revealed that the tumours were of mixed cell sarcoma-type with a considerable angiomatous component, similar to those described for *wt* AEV². Also in agreement with observations of *wt* AEV, no metastases were found in any of the birds (we examined liver, heart, kidneys and lungs).

The observed tumour specificity of *td359* AEV is reminiscent of that of RSV and raises the question of whether or not we were dealing with an RSV contaminant. This is unlikely for the following reasons. First, as described above, cells transformed

by *td359* AEV exhibit a transformation phenotype characteristic of AEV-transformed cells. Second, non-producer cells transformed by *td359* AEV did not contain any of the structural proteins of RSV, when tested by radioimmunoprecipitation using high titre anti-virus sera (our unpublished results). Instead, they contained the AEV-specific protein P75 (ref. 4) in an altered form¹⁰. Third, AEV-specific RNA sequences were detected in a cloned pseudotype of the mutant (S. Saule and D. Stéhelin, personal communication).

Table 4 *In vivo* assay of *td359* AEV

Type of inoculum and route of inoculation	Incidence of animals with	
	erythroblastosis	sarcomas
<i>wt</i> AEV (RAV-1) virus, i.v.	5/5	0/5
<i>td359</i> AEV (RAV-1) virus, i.v.	0/10	1/10
<i>td359</i> AEV (RAV-1)-transformed fibroblasts, i.m.	0/6	6/6

The isolation of an AEV mutant of the *td359* type could indicate that there are two separate genes for transformation of erythroblasts and of fibroblasts. From the complexity of the AEV-specific sequences of about 3,700 nucleotides¹², this would be possible. On the other hand, the finding that *ts34* AEV, a mutant isolated in the bone marrow system⁹, is not only temperature-sensitive for erythroblast transformation but also affected in the expression of some transformation parameters in fibroblasts at the nonpermissive temperature¹⁰, suggests that one gene is needed for both functions. It will be necessary to isolate more *td359*-type mutants and further *ts* mutants to settle the question of whether one or more genes are required for leukaemic and sarcomagenic transformation. It would be interesting to know if AEV mutants can be isolated that are deficient for sarcoma formation but able to transform erythroblasts.

Studies are in progress in collaboration with Stéhelin's group, to determine whether *td359* AEV has a deletion in the oncogene of AEV, termed *erb*^{7,10,13,14}. Hanafusa *et al.*¹¹ have shown that the sarcomagenicity of RSV mutants with a partial deletion in the *src* gene can be restored by injecting them into animals. This is possible because sequences homologous to the *src* gene have been found in the DNA of normal cells¹². Stéhelin and his group have demonstrated that sequences homologous to the *erb* gene of AEV also exist in cellular DNA^{7,13}. It would therefore be interesting to determine whether the leukaemogenic potential of *td359* AEV can be restored by passaging the virus through animal hosts.

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Isolation of a replication-defective murine leukaemia virus from cultured AKR leukaemia cells

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Mice of the AKR strain are characterised by a high incidence of spontaneous thymic lymphomas¹. AKR chromosomes contain the genomes of ecotropic murine leukaemia virus (MuLV) at two loci, termed *Akv-1* and *Akv-2* (refs 2–6). Shortly after birth, the normal tissues of AKR mice begin to produce high levels of this XC-positive MuLV (ref. 7) (that is, one that forms XC plaques). A second class of MuLV, termed mink cell focus-inducing virus (MCF), is produced specifically by preleukaemic and leukaemic AKR thymocytes^{8,9}. Nowinski *et al.* have established a series of tissue culture lines from AKR leukaemias^{10,11} and reported that the resulting cell lines produce virus particles, but that these particles, surprisingly, do not give rise to XC plaques. We have analysed the virus particles produced by one of these cell lines, termed AKRSL2. We show here that, unlike most or all of the nonmalignant tissues in the AKR mouse⁷, these cultured lymphoma cells produce very little non-defective ecotropic MuLV; however, they do produce replication-defective ecotropic MuLV.

Nowinski *et al.* reported that when normal mouse fibroblasts are first infected with the virus particles produced by the AKR leukaemia cell lines, and then passaged for an extended period, they ultimately release MuLV capable of forming XC plaques¹⁰. As we have noted, this delayed production of XC-positive MuLV is characteristic of infection by particles containing replication-defective ecotropic MuLV genomes¹². Particles of this type can readily be detected by using XC-negative competent MuLVs as 'helper' viruses; thus, infectious particles of replication-defective ecotropic MuLV, which cannot form plaques in the standard XC test, do give rise to XC plaques (termed 'complementation plaques') if the assay cells are co-infected with an XC-negative, competent MuLV^{13,14}. In our initial experiments, culture fluids of AKRSL2 cells were tested for infectious MuLV by three different assays: the S+L- focus assay¹⁵, which detects all known MuLVs capable of replicating themselves in mouse cells ('competent' MuLVs); the standard XC test¹⁶, which specifically detects competent ecotropic MuLVs; and the complementation-plaque assay, which detects replication-defective, as well as competent, ecotropic MuLVs¹³. It was found (Table 1a) that AKRSL2 culture fluids contain approximately 10^3 focus-inducing units (FIU) per ml of competent MuLV. This MuLV is not standard ecotropic MuLV, however, since, in agreement with the results of Nowinski *et al.*¹⁰ and Table 1a, the same fluid contained virtually no MuLV which formed XC plaques. The identity of this XC-negative competent MuLV will be considered further below. Finally, approximately 2×10^3 particles per ml which register in the complementation-plaque assay (termed complementation-plaque forming units or CPFU) were also present in these fluids (Table 1a). The CPFU appear to represent infectious particles of replication-defective ecotropic MuLV, since they are detected in the complementation-plaque assay but not in the standard XC test.

It was also found (Table 1b) that cell clones isolated from the AKRSL2 cell line all produce both competent XC-negative MuLV and replication-defective ecotropic MuLV at titres similar to those produced by mass cultures of AKRSL2 cells. Thus AKRSL2 cells appear to be a homogeneous population with respect to virus production.

Table 1 Production of infectious MuLV by AKRSL2 cells and by clones of AKRSL2 cells

Cell line ^a	Assay		
	S+L- focus assay (FIU ml ⁻¹)	XC test (PFU ml ⁻¹)	Complementation-plaque assay (CPFU ml ⁻¹)
AKRSL2	1×10^3	4×10^1	2×10^3
Clone ^b			
1	2×10^2	$<1 \times 10^1$	1×10^4
2	3×10^3	$<1 \times 10^1$	4×10^4
3	1×10^3	$<1 \times 10^1$	1×10^4
4	3×10^3	$<1 \times 10^1$	2×10^4
5	4×10^3	$<1 \times 10^1$	2×10^4

a, AKRSL2 cells were grown in RPMI-1640 medium with 10% heat-inactivated fetal calf serum (GIBCO). Clarified AKRSL2 culture fluids were assayed on S+L- cells as described¹⁵. The XC test¹⁶ was performed on 3T3FL cells as described¹³. The complementation-plaque assay¹³ differs from the XC test only in that the assay cells are simultaneously infected with the test virus and $1.3 \text{ FIU cell}^{-1}$ of Moloney clone 83 (obtained from D. Troxler and propagated in 3T3FL cells). All other reagents and procedures were as described¹²⁻¹⁴. b, AKRSL2 cells were cloned by seeding 0.2 cells per well in microtest plates (Costar). Each clone was grown to mass culture and tested in a.

Since AKRSL2 supernatants appear to represent a mixture of two different types of MuLV (Table 1), it seemed crucial to separate these two viruses from each other, so that they could be analysed more definitively. We therefore tried to transmit them to other cells in conditions of single infection. 3T3FL cells were infected at a low multiplicity of infection (MOI) with the culture fluid of AKRSL2 clone 2, and cloned the following day; each clone was then screened for the presence of competent MuLV and of rescuable, replication-defective ecotropic MuLV. Several clones were isolated which appeared to contain defective ecotropic MuLV in the absence of competent MuLV; one of these clones, termed AK24, is described below.

Clone AK24 produces no infectious MuLV capable of forming either S+L- foci or complementation plaques (Table 2a). However, when this clone is superinfected by an XC-negative competent virus, such as amphotropic MuLV¹⁷⁻¹⁹, Moloney clone 83 (ref. 20), MCF 247 (ref. 9) or the supernatant of clone AK45 (see below), it releases CPFU of ecotropic MuLV as well as FIU of competent MuLV (Table 2a).

The appearance of CPFU when clone AK24 cells are superinfected with competent MuLV could represent the rescue of a defective virus; alternatively, these CPFU might be competent particles of ecotropic MuLV, formed as a result of recombination between the defective MuLV in clone AK24 cells and the superinfecting virus. In tests to distinguish between these possibilities, it was found that (1) supernatants of superinfected AK24 cells form plaques with a 2-hit dose-response curve in the standard XC test, and (2) when fresh cells are infected at low MOI with CPFU from superinfected AK24 cells, they in turn require superinfection with a competent 'helper' virus for production of infectious CPFU (data not shown). These results are analogous to those in a previous study of a replication-defective variant of Moloney MuLV¹⁴.

Taken together, these results demonstrate that the ecotropic MuLV in clone AK24 cells is replication-defective: it produces no infectious progeny on solitary infection, and is rescued as infectious, replication-defective ecotropic MuLV particles when the cells are also infected with a competent, 'helper' MuLV.

One clone isolated in the same experiment as AK24, termed clone AK45, differs from clone AK24 in that it produces

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competent, XC-negative MuLV, but does not produce CPFU, even after superinfection with helper viruses (Table 2b). Clone AK45 thus appears to be infected with the competent MuLV, but not with the defective ecotropic MuLV, produced by AKRSL2 cells. The fact that this competent MuLV is XC-negative suggests that it may be an isolate of MCF⁹. In preliminary tests, this virus has not induced detectable foci in mink cells, but appears to be related to MCF by viral interference tests (data not shown). This MuLV is capable of rescuing CPFU from clone AK24 (Table 2a); it seems likely that it is the 'helper' for the defective ecotropic MuLV in AKRSL2 cells.

It was of interest to analyse the defectiveness of the MuLV in clone AK24 cells. When thin sections of these cells were examined in the electron microscope, no particles or clear virus-specific structures were seen (G. Schidlovsky, personal communication). Clone AK24 supernatants also contained no detectable particle-associated reverse transcriptase activity (data not shown). Several other clones isolated in the same experiment as AK24 also contain rescuable CPFU (as in Table 2a) but produce no virus particles (data not shown). Thus the defective ecotropic MuLV genome present in AKRSL2 cells apparently does not code for all of the viral structural proteins required for virus particle assembly. (Nowinski *et al.* noted that fibroblasts infected with AKRSL2 supernatant produce XC-positive MuLV on extended passage¹⁰. We have not observed virus production in clone AK24 cultures; thus the 'conversion' of the defective to XC-positive MuLV may require co-infection with the competent XC-negative MuLV).

Table 2 Transmission of replication-defective ecotropic MuLV and XC-negative competent MuLV from AKRSL2 cells to 3T3FL cells

Superinfecting MuLV ^a	CPFU (ml ⁻¹)	FIU (ml ⁻¹)
None	<1 × 10 ⁰	<1 × 10 ⁰
Amphotropic MuLV	2 × 10 ⁴	3 × 10 ⁴
Moloney clone 83	2 × 10 ⁴	1 × 10 ⁵
MCF 247	7 × 10 ¹	2 × 10 ²
Clone AK45 MuLV	3 × 10 ²	8 × 10 ²
Superinfecting MuLV ^b	CPFU (ml ⁻¹)	FIU (ml ⁻¹)
None	<1 × 10 ⁰	1 × 10 ⁴
Amphotropic MuLV	<1 × 10 ⁰	2 × 10 ⁵
Moloney clone 83	<1 × 10 ⁰	5 × 10 ³

a, 3T3FL cells were infected with AKRSL2 clone 2 culture fluid at a MOI of 0.13 CPFU per cell, and were then cloned in Microtest plates. Nineteen clones were screened for the presence of rescuable ecotropic MuLV by testing their ability to fuse XC cells after co-cultivation for 5 d with 3T3FL cells producing Moloney clone 83; they were also screened for competent MuLV production by testing the ability of mitomycin C-treated cells to induce morphological alterations in S+L- cells¹⁵. From these tests four clones, including clone AK24, appeared to contain a rescuable ecotropic MuLV, but no competent MuLV. Virus production by clone AK24 was then tested directly as follows. Clone AK24 cultures were mock-infected or superinfected with amphotropic MuLV (4 FIU per cell), Moloney clone 83 (8 FIU per cell), MCF 247 (obtained by J. Hartley and propagated in 3T3FL cells) (0.04 FIU per cell), or the supernatant of clone AK45 (0.2 FIU per cell) (see below). Five days after infection, the supernatants of these cultures were collected and assayed for CPFU and FIU as described¹³⁻¹⁵. *b*, 3T3FL cells were infected with AKRSL2 clone 2 culture fluid at a MOI of 0.7 CPFU per cell, and were cloned and tested as described in *a* above. Virus production by clone AK45 was then tested as described in *a*.

In summary, AKRSL2 cells, originally described as producing 'XC-negative' MuLV^{10,11}, actually produce a replication-defective ecotropic MuLV and an XC-negative, competent MuLV; these two MuLVs can be detected by appropriate *in vitro* assay techniques (Table 1), and can be dissociated from each other by virus cloning procedures, that is, infection of fresh host cells at low MOI followed by cell cloning (Table 2). It will be important to determine whether other AKR leukaemia lines also produce these two types of MuLV.

The findings reported here raise several new questions. Since AKRSL2 cells would be expected to contain the two loci,

Akv-1 and *Akv-2*, which code for competent ecotropic MuLV²⁻⁶, it is remarkable that they do not produce such MuLV. What is the origin of the defective ecotropic MuLV produced by AKRSL2 cells? Is it the product of a third endogenous viral locus^{21,22}, or does it arise by modification of *Akv-1* and/or *Akv-2* during the lifetime of the mouse (or in culture)? Further, since virtually all non-malignant AKR tissues produce competent ecotropic MuLV⁷, is there a connection between the defectiveness of the ecotropic MuLV in AKR leukaemia cells and the malignancy of these cells? For example, might some of the information for viral structural proteins in *Akv-1* or *Akv-2* have been replaced by leukaemogenic information? In this regard, Nowinski *et al.* reported^{10,11} that AKRSL2 culture fluid can accelerate the onset of leukaemia when injected into newborn AKR mice. We have shown here that this fluid contains two distinct viruses; experiments are now in progress to determine which of these viruses accelerates leukaemia development.

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Replicative form of Semliki Forest virus RNA contains an unpaired guanosine

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The mechanism of replication of the genome of animal viruses containing infectious single-stranded RNA is not understood in detail. Alphaviruses contain an infectious single-stranded genome RNA of about 4.2×10^6 molecular weight, which sediments at about 42S on sucrose density gradients¹⁻⁴. Accordingly they are designated as (+)strand RNA viruses⁵. Most studies on the structure or replication of alphaviruses have

been made using either Sindbis (SIN) or Semliki Forest (SF) viruses. Besides the infectious 42S plus-strand RNA, a 26S single-stranded RNA of positive polarity is synthesised in alphavirus-infected cells^{3,4,6}. The 26S RNA sequences are identical to the sequences present in the 3'-terminal region of the 42S RNA^{7,8}. The 42S RNA functions as mRNA for the synthesis of nonstructural proteins including an RNA polymerase⁹⁻¹¹. The 26S RNA is the mRNA for all viral structural proteins¹²⁻¹⁵. The 42S SIN virus genome RNA has the 5'-terminal structure m⁷GpppApUpYpGp (ref. 16), and a cap structure containing m₁⁷G, m₂⁷G or m₃⁷G is present in SIN virus-specific 26S RNA (ref. 17). Analogous studies on the 5'-termini of the corresponding SF-virus-specific nucleic acids have not been reported. The only species of virus-specific RNA of negative polarity detected in alphavirus-infected cells sediments at about 42S on sucrose density gradients and is complementary to the infectious viral genome RNA¹⁸⁻²¹. The poly(A) sequences present at the 3'-termini of the virus-specific 42S and 26S RNA molecules²²⁻²⁴ are transcribed from a complementary poly(U) sequence²⁰. We now report that we have identified the structures of cap containing oligonucleotides of the SF virus-specific (+)strand RNAs, and the sequence of 23

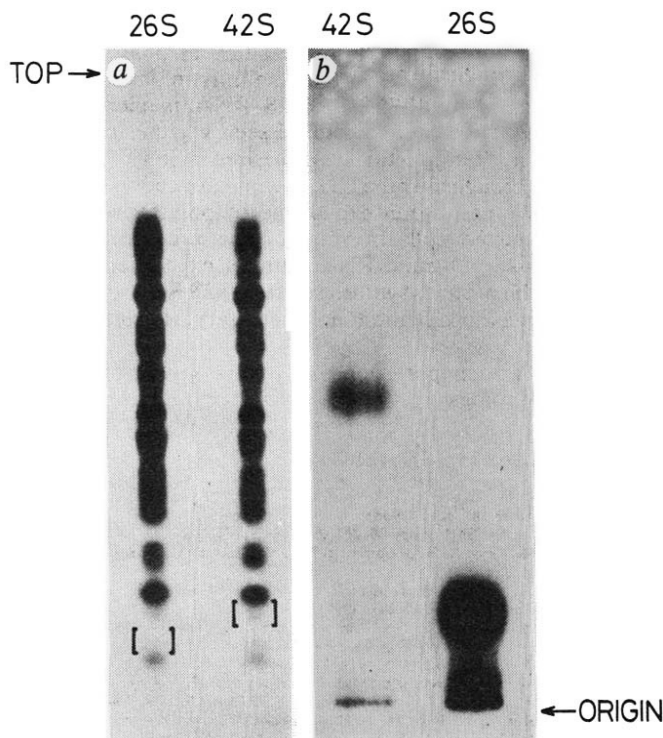


Fig. 1 Purification of cap containing oligonucleotides. ³²P-labelled alphavirus-specific 26S and 42S (+)strand RNA was isolated from Semliki Forest virus-infected BHK cells by phenol extraction and sucrose density gradient centrifugation⁷. The labelled RNA was completely digested by ribonuclease T₁ and the cap containing oligonucleotides were purified by polyacrylamide gel electrophoresis at pH 3.5 (ref. 27). *a*, Each lane was divided into 1-cm sections, the oligonucleotides of each fraction were eluted with 500 mM triethylamine acetate, pH 6.0, containing 50 μg ml⁻¹ of carrier tRNA and precipitated at 25 °C after addition of 3 vol of ethanol. An aliquot of each fraction was digested with RNase T₂ (ref. 28) and the products were analysed by electrophoresis on DEAE-paper at pH 3.5 (ref. 29) (data not shown) for the slow-moving cap structure m⁷GpppAp. These corresponding fractions are indicated by vertical bars in *a*. The capped oligonucleotide present in either of these fractions was subjected to borate-cellulose chromatography^{30,31} (data not shown) and was further purified by electrophoresis on DEAE-paper in 7% formic acid pH 1.7 (*b*). The difference in electrophoretic mobility between both oligonucleotides indicates that the more slowly moving one presumably contains one Up nucleotide more than the faster one²⁹.

in infected cells. Our studies show that the replicative form of SF virus contains an unpaired guanosine at the 3'-end of the (-)strand.

As can be seen from the data presented in Fig. 1, the RNase T₁-resistant cap containing oligonucleotides of SF-virus-specific 42S and 26S RNA behave differently during electrophoresis at pH 3.5 as well as at pH 1.7. The structure of both oligonucleotides was determined as shown in Fig. 2. For both RNAs m⁷GpppA caps were found (see Fig. 2*a* for the cap derived from 42S RNA). Analyses of the digestion products obtained with pancreatic RNase (Fig. 2*b*), together with the differences in electrophoretic mobility shown in Fig. 1*b*, demonstrate that the 5'-terminal structures are m⁷GpppApUpGp for 42S RNA and m⁷GpppApUpUpGp for 26S RNA, respectively.

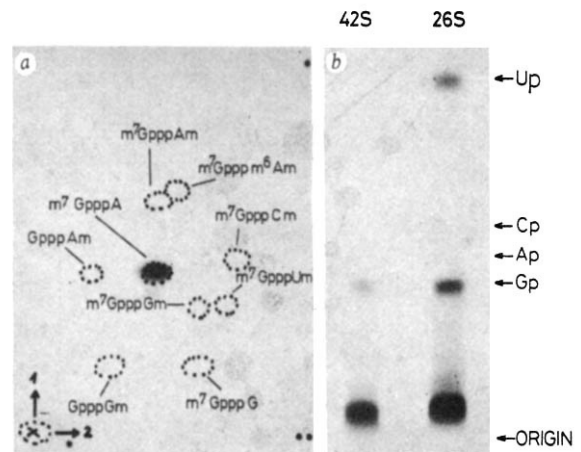


Fig. 2 Analyses of ³²P-labelled cap-containing oligonucleotides. Cap-containing oligonucleotides of SF virus-specific 42S and 26S RNA, purified as described in Fig. 1 legend, were eluted from the DEAE-paper after electrophoresis and analysed after RNase digestions. RNase T₂ yielded m⁷GpppAp, Up and Gp for both cap-containing oligonucleotides (not shown). The products of combined RNase T₂ and phosphatase treatment were separated from ³²P-phosphate by a short electrophoresis on DEAE-paper, pH 1.7. After autoradiography and elution, the RNase T₂ and phosphatase-resistant material was identified by two-dimensional co-chromatography^{28,32} with authentic compounds (dotted circles) as m⁷GpppA in either case. *a*, Identification of the 42S RNA cap. Digestion of the cap-containing oligonucleotides with pancreatic RNase and cellulose TLC (solvent: t-butanol/conc. HCl/H₂O = 7:1.5:1.5, v/v/v) yielded Gp (*b*, left lane) or Gp plus Up (*b*, right lane) in addition to m⁷GpppApUp.

The mode of synthesis of 26S RNA in alphavirus-infected cells has not been precisely determined. Since the only species of virus-specific RNA of minus polarity found in alphavirus-infected cells is the 42S (-)strand RNA described above¹⁸⁻²¹ it must be concluded that this RNA is the template for the synthesis of both the 26S and 42S RNAs of positive polarity. The finding that the 5'-terminal sequences of the SF virus-specific 42S and 26S (+)strand RNA molecules are different indicates that the 26S RNA is not generated from a 42S RNA intermediate by a splicing process.

A further characterisation of the 5'-terminal sequence of the 42S (+)strand RNA was possible by determination of the sequence of 23 nucleotides at the 3'-terminus of the complementary 42S (-)strand RNA (Fig. 3). This sequence can be aligned with the complementary m⁷GpppApUpGp of the 42S (+)strand RNA and allows one to derive a complementary sequence of 22 nucleotides at the 5'-terminus of the latter molecule.

The surprising outcome of this alignment is shown in Fig. 4: there is an extra unpaired guanosine at the 3'-end of SF virus (-)strand 42S RNA. It is important to point out here that this result was obtained with the replicative complex of (+) and (-)strand 42S RNA as isolated directly from the infected cell,

since the detection of an unpaired guanosine at the 3'-end of SF virus (-)strand RNA is the first observation of this type in an eukaryotic RNA virus.

SF virus-specific 42S (-)strand RNA contains a poly(U) sequence at its 5'-terminus, which on average is probably somewhat shorter than the corresponding 3'-terminal poly(A) sequence of the 42S (+)strand RNA²⁰. This, with our results, indicates that the 3'-ends of both these 42S RNA molecules are neither used as templates for the synthesis of complementary RNA nor generated by transcription of a complementary RNA sequence. It is possible that both unpaired structures act as polymerase recognition sites during the initiation of viral RNA

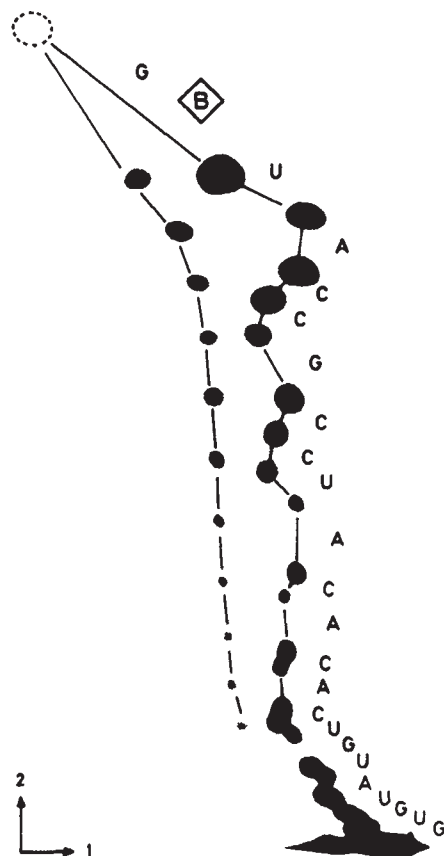


Fig. 3 Sequence analysis of [$5'$ - ^{32}P]pCp labelled SF virus-specific double-stranded RNA. An unlabelled 22S double-stranded complex consisting of 42S RNA of both positive and negative polarity was isolated from SF virus-infected BHK cells²¹, heat denatured, and labelled at the 3'-termini of the constituent molecules with [$5'$ - ^{32}P]pCp using RNA ligase³³. The labelled RNA was hybridised to an excess of unlabelled 42S (+)strand RNA and the complex RNA generated during hybridisation was purified by CF 11-cellulose chromatography³⁴ and subjected to partial alkaline fragmentation³⁵. 5×10^4 c.p.m. with 25 μg carrier tRNA were dissolved in 20 μl 50 mM NaHCO_3 , 0.5 mM EDTA, pH 9.1 and kept in boiling water for 20 min. The resulting oligonucleotides were fractionated by cellulose-acetate electrophoresis at pH 3.5 (first dimension) followed by homochromatography on DEAE-cellulose coated thin layer plates (second dimension) in 10 mM Homomix, pH 4.7 (refs 36, 37). An autoradiogram of this analysis is shown. The sequence of nucleotides was determined by the mobility shift between successive spots³⁶. The sequence in the lower unresolved region of the autoradiogram was established in a second experiment by more extended homochromatography. A (5')... CpApUpGp(3') sequence resulting from fragments which had lost the 3'-terminal Cp, was detectable in overexposed autoradiograms, thus providing additional evidence for the sequence. The poly(A) sequence visible on the left side of the autoradiogram derives from contaminating 3'-end labelled (+)strand RNA. The dotted circle indicates the location of the ligated 3'-terminal Cp.

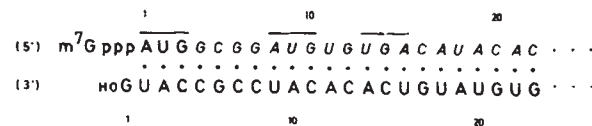


Fig. 4 Structure at the 5'-end of (+) and at the 3'-end of (-)strand RNA of SF virus-specific replicative form. The sequence of 23 3'-terminal nucleotides of the (-)strand (bold letters) was determined by analysis of *in vitro* end-labelled material (Fig. 3). The m⁷GpppApUpG fragment of the (+)strand (bold letters) was characterised from *in vivo* labelled RNA (Fig. 2). Nucleotides 4-22 of the (+)strand (smaller letters) were derived from the established (-)strand sequence. Bars above the (+)strand indicate the location of possible initiation and termination signals for protein synthesis. Both RNA sequences shown above can be folded into several secondary structures of marginal stability.

replication; however, this, as well as their mode of synthesis, remains to be determined.

The available data concerning the localisation of initiation triplets on eukaryotic mRNA indicate that translation starts at the 5'-proximal AUG codon²⁵. If translation of the 42S (+)strand RNA started at the first AUG triplet, only the tetrapeptide Met-Ala-Asp-Val would be generated, due to the presence of the termination codon UGA in phase (Fig. 4). Glanville *et al.*²⁶ have shown that the only initiation peptide synthesised on SF virus-specific 42S RNA *in vitro* has the structure Met-Ala. It remains to be seen whether or not the abovementioned tetrapeptide is synthesised in SF virus-infected cells. A readthrough over the UGA terminator codon leading to polymerase synthesis would explain the low polymerase levels in alphavirus-infected cells in spite of the presence of large amounts of 42S (+)strand RNA. Further experiments on the structure of the proteins synthesised from 42S RNA *in vivo* and more sequence information are necessary to clarify these points.

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matters arising

The interpretation of tree-ring $\delta^{13}\text{C}$ records

RECENTLY Farmer¹ reported on the decline of $\delta^{13}\text{C}$ in the tree rings of the American elm from 1883 to 1933 and generally supports the net biospheric CO_2 release theory due to the so-called pioneer agricultural expansion of the late nineteenth century. Other data from various parts of the Northern Hemisphere show a similar decline in the $^{13}\text{C}/^{12}\text{C}$ ratio in tree rings²⁻⁵. However, the tree-ring records produced after 1930 are less uniform in trend, with the American elm indicating a dramatic increase in $\delta^{13}\text{C}$ from 1930 to 1970. Several explanations of this increasing trend have been put forward, including a cessation of the net biospheric CO_2 flux; but as Farmer notes, more complicated interactions may be at work. I wish to point out that these investigations have ignored the shift in the fossil fuel consumption pattern of coal, petroleum and natural gas during the period 1850–1970 which may have a significant effect on atmospheric $\delta^{13}\text{C}$.

From data presented by Stuiver⁴ and Wedepohl and Althaus⁶, I have taken $\delta^{13}\text{C}$ fossil fuel values of -24.5% (coal), -28.5% (oil) and -41.5% (gas) and multiplied the appropriate $^{13}\text{C}/^{12}\text{C}$ ratios by the per cent production estimates provided by Rotty⁷ for 1950–74 and Dorf⁸ for pre-1950 data. The result of this calculation, an estimate for the $\delta^{13}\text{C}$ of fossil

fuel produced CO_2 as a time function which appears in Fig. 1, is to modify the equation for computing the change in atmospheric $\delta^{13}\text{C}$ caused by fossil fuel CO_2 used by Stuiver⁴ and Farmer¹ from

$$\Delta(\delta^{13}\text{C}) = 0.18S \quad (1)$$

to

$$\Delta(\delta^{13}\text{C}) = (\gamma/100)S \quad (2)$$

where γ takes the values of Fig. 1 less 7 (the per mil atmospheric $\delta^{13}\text{C}$) and S is the per cent ^{14}C change.

Equation (1) is a good approximation for the $\delta^{13}\text{C}$ change before 1930; however, by 1970, when the fossil fuel $\delta^{13}\text{C}$ declines to -29 per mil, a 22% error is incurred by not using equation (2). Note that this error amplifies the unexplained upward trend of the tree-ring data after correction for the fossil fuel CO_2 atmospheric input. Although the $\delta^{13}\text{C}$ upward trend (Fig. 1c of ref. 1) may be correlated with a local¹, and perhaps global⁹, temperature decrease during the post-1930 period, this effect may also be interactive. The biospheric CO_2 input of 120 BMT (10^9 metric tonnes = 1 BMT) computed by Stuiver⁴ and Wilson⁵, if sufficiently concentrated around the year 1900, could cause a temperature effect of the magnitude and timing noted in the global records for the Northern Hemisphere⁹.

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0.47% to 0.54% by 1960. These alterations are insignificant in the context of correcting the observed tree-ring $\delta^{13}\text{C}$ variations for the effects of fossil fuel CO_2 input on atmospheric $\delta^{13}\text{C}$ (Fig. 1a, c of ref. 1) and suggest that the use of 18 for γ is a good approximation to at least 1950.

Although the alteration would be more marked by 1970, amounting to a change of 0.16% from 0.70% to 0.86%, the net result of all such alterations, as Mulholland observes, would in any case be the enhancement of the upward trend since 1933 of the fossil-fuel CO_2 corrected $\delta^{13}\text{C}$ curve for the American elm (Fig. 1c of ref. 1). Thus the apparent 'fossil-fuel CO_2 corrected' $\delta^{13}\text{C}$ -atmospheric temperature correlation for the elm post-1933 remains. The need for investigation of tree-ring $^{13}\text{C}/^{12}\text{C}$ ratios before the onset of major anthropogenic activity, to aid resolution of perturbatory parameters since 1850, is re-emphasised.

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Oxidation-reduction potentials of haem proteins

STELLWAGEN¹ has compared oxidation-reduction potentials of seven haem proteins with respect to parameters of haem accessibility and polarity of the haem crevice calculated on the basis of known three-dimensional structures.

One point, however, has been overlooked. Kassner² presented a model not for estimation of whole E'_0 , but only for an increment of E'_0 (assigned as $\Delta E'_0$) which must be added to the E'_0 value of the cytochrome *c* model system to obtain values which correspond better to the experimental ones. Kassner's equation for $\Delta E'_0$ expresses the fact that greater thickness and apolarity of the haem apolar environment leads to higher values of the increment $\Delta E'_0$.

The values of $\Delta E'_0$ should then be added to E'_0 for haem protein models. For the

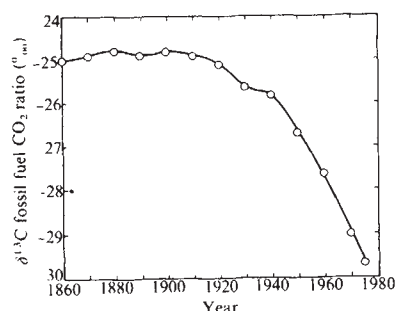


Fig. 1 Estimate of ^{13}C content of combusted fossil fuels with respect to their historical use pattern. The change in relative deficiency beginning in the early part of this century and continuing to the present indicates the shift from coal to petroleum and later to natural gas. This curve resolves part of the deficiency in ^{13}C in tree-ring data due to fossil fuel combustion.

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FARMER REPLIES—The insertion of a varying $\delta^{13}\text{C}$ of fossil fuel CO_2 (Fig. 1) in equation (2), as proposed by Mulholland, would alter the estimated decline in atmospheric $\delta^{13}\text{C}$ due to fossil fuel CO_2 input (Fig. 1b of ref. 1) by 0.01% from 0.28% to 0.29% by 1940, 0.03% from 0.35% to 0.38% by 1950 and 0.07% from

complexes with histidine and methionine as axial haem ligands (as in cytochromes *c*) values for uncorrected E'_0 of about -0.10 V have been obtained^{3,4}, with one histidine (as in haemoglobins) at about -0.16 V and with two histidines (analogous to cytochromes *b*) at -0.21 V. Thus, for different haem proteins, different corrected E'_0 values are obtained when the same value of increment $\Delta E'_0$ is added.

Moreover, from the viewpoint of haem accessibility, the lower $\Delta E'_0$ corresponds, according to Kassner², to the better accessibility of haem. Stellwagen¹ presented the data of haem accessibility (cytochromes *c* about 5% haem exposed, haemoglobin subunits ~16–18%, cytochrome *b*₅ ~23%). Thus, the highest values of E'_0 must be obtained for the cytochromes *c* because of both high E'_0 of model system and high $\Delta E'_0$; and lowest for the cytochrome *b*₅ (the lowest values of E'_0 and $\Delta E'_0$). This is, however, in good correlation with the experimental values of E'_0 as presented by Stellwagen¹ (average 0.280 V for cytochromes *c*, 0.080 V for haemoglobin subunits and 0.020 mV for cytochrome *b*₅).

Thus, it is most probable that both the effect of haem axial ligands and the nature of haem surroundings determine the actual value of oxidation–reduction potential. This may be crucial in discussing, for example, the high negative values of E'_0 of cytochromes P-450 (ref. 5), where one of the axial ligands is presumably the mercaptide (anionic) sulphur⁶.

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STELLWAGEN REPLIES—Anzenbacher correctly points out that the Kassner proposal concerns the $\Delta E'_0$ resulting from complexation of haem by apoprotein without changing the nature of the axial ligands and suggests that the E'_0 values for the haem octapeptide from cytochrome *c* having histidine in the fifth coordination position and either water, imidazole acetylmethionine in the sixth coordination position would serve as appropriate model haems for the haemoproteins considered. However, for purposes of surface area calculation it should be recognised that the atoms of the octapeptide very likely

shield part of the haem from the solvent owing to the three point attachment of haem to octapeptide (two thioether bridges and one coordinate:covalent bond). While the surface area of a haem coordinated with either one or two histidines or one histidine and one methionine could be calculated by elimination of the remaining protein atoms in the crystallographic models of appropriate haem proteins, the E'_0 values of these coordinated haems would not necessarily be identical with E'_0 of the octapeptide models owing to potential differences in the geometry of the axial coordinate:covalent bonds. However, assuming that the E'_0 values of the octapeptide models are appropriate and that the haem exposure in each model is the same, recalculation of Fig. 2 of my paper $\Delta E'_0$ values yields essentially the same relationships. In any case, the major point remains, namely that the apolarity of the haem crevice or of total haem environment seems independent of E'_0 or $\Delta E'_0$ for haemoproteins having either one or both axial ligand identical.

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Effect of chloroquine on Epstein–Barr virus expression

THE finding by Karmali *et al.*¹ that 'chloroquine enhances Epstein–Barr virus expression' was of great concern to us as it implies that chloroquine, through its effect on the virus, might stimulate the development of Burkitt's lymphoma (BL). We are using chloroquine to suppress malaria in a child population in Tanzania in an attempt to reduce the incidence of BL. A BL-promoting effect of chloroquine might tend to mask any reduction in BL incidence brought about by malaria suppression and would greatly complicate the interpretation of the findings in our trial.

We have therefore repeated and extended Karmali's experiments in our laboratory. This type of experiment is frequently carried out by us, as studies on the expression of Epstein–Barr virus (EBV) antigens is one of our major topics of interest². We found that, as Karmali reported, chloroquine had no effect on spontaneous expression of EBV antigens in either non-producer (Raji) or producer (P3HR-1 or B95) lines. We also found no effect of this compound on chemical induction of early antigen (EA) in Raji cells. Furthermore, in an attempt to repeat Karmali's experiment using superinfected Raji cells, we were unable to reproduce his results.

In our hands, whatever the multiplicity of infection, dose or origin of chloroquine used (from different commercial firms, including the one used in Karmali's experiment), we found no effect of chloroquine on expression of EBV antigens. We therefore feel that Karmali's conclusions should be reconsidered, for even if it were true that expression of EBV antigens could be enhanced by chloroquine in the system he describes, this system is very particular, as the P3HR-1 EB virus is a unique laboratory 'strain' which has lost one of the major properties of all other EBV isolates, its transforming ability. Moreover, in all other systems of EBV antigen synthesis studied, chloroquine has no effect.

In the absence of significant effects of chloroquine on EBV expression in the laboratory and as no effect of chloroquine medication on EBV serology has ever been reported in the many populations who receive chloroquine for malaria prophylaxis, we are convinced that Karmali's conclusions regarding the effect of chloroquine on BL development are premature. We are, therefore, continuing our studies of malaria suppression and BL incidence, as originally planned.

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KARMALI AND HORROBIN REPLY—We have reviewed our work carefully. The observations were made blind by three separate individuals and we stand by our findings. Minor differences of technique can lead to different results and we have no explanation for the discrepancy. We agree with Lenoir and Geser that the chloroquine trial should not be stopped because of a laboratory observation, for such observations in highly artificial conditions may be poor guides to *in vivo* results. Nevertheless, our work does sound a note of caution and we would argue that the trial should be monitored exceptionally carefully and any evidence of increased rather than reduced Burkitt lymphoma incidence investigated immediately.

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reviews

Elementary, my dear Eiseley

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Darwin and the Mysterious Mr X: New Light on the Evolutionists. By Loren Eiseley. Pp.278. (Dutton: New York; Dent: London, 1979.) \$12.95; £6.95.

ANYTHING written by the late Loren Eiseley is worth reading, but some of his books and articles more than others. This book is a reprint of various essays which originally appeared in periodicals from 1956–72. Those on “Charles Darwin” (1956), “Alfred Russel Wallace” (1959) “Charles Lyell” (1959), “Charles Darwin”, Edward Blyth, and the Theory of Natural Selection” (1959) and “Darwin, Coleridge and the Theory of Unconscious Creation” (1965) form Part I. Part II is made up of three articles by Edward Blyth, originally published in 1835, 1836 and 1837, and Part III is a useful reprint of Arthur Grote’s memoir of him (1975); both these parts were originally reprinted as appendices to the essay on Darwin and Blyth. Lastly, Part IV has three delightful if not very profound essays on the evolution of Man: “Neanderthal Man and the Dawn of Human Paleontology (1957); “Intellectual Antecedents of The Descent of Man” (1972), and a meditation on Man’s capacity for self-destruction, “The Time of Man” (1962).

Both the editor and the publishers single out the essay on Darwin and Blyth as Eiseley’s major contribution in this collection to the history of Charles Darwin’s intellectual development. The editor says, “Here Eiseley argues that the basic tenets of the theory of natural selection were stated by Blyth much earlier than by Darwin, and that Darwin was familiar with Blyth’s idea, made use of it in his theories, and yet failed to acknowledge his obligation to Blyth”, and he comments that without the documentary evidence (the reprints of Blyth’s papers) “his conclusions would be almost incredible”. Eiseley himself says he was forced “to call upon all the skills of the literary detective” to make out his case, and apparently first thought of writing it up as a detective story (hence the title, taken from his incomplete manuscript). But, according to his editor, he realised that to be effective, he would have to publish his account “in a scholarly journal”. I do not think that this consideration was kept firmly enough before his eyes.

Eiseley’s argument is that Darwin used a rare word, “inosculate”, which is found also in Blyth, and that although he gives much credit to Blyth for information, he carefully avoided referring to a group of



Edward Blyth

Blyth’s papers in which the word occurs, and in which, to Eiseley’s surprise, a nearly complete theory of evolution by natural selection is to be found. Moreover, in Darwin’s early notebooks, now published, there is such a resemblance between topics and expressions in certain passages and these papers of Blyth’s that Darwin must have read them (which indeed, no-one need doubt), and they were read at a time when Darwin rapidly changed his thinking, abandoning his earlier idea that species could only form by jumps, and going over to their gradual transformation. The papers had therefore a great influence on him which he should have acknowledged. Darwin was notoriously slow and reluctant to publish a historical sketch doing justice to his predecessors, and here is a further case where he omitted to make due acknowledgement, from a very human desire to keep his great contribution as his own. Eiseley’s essay on Coleridge is to show (via John Livingston Lowes) that the production of *Kubla Khan* was a highly conscious combination of pre-existing material, not an unconscious creation in the mind of genius, and so must Charles Darwin’s great work have been. Fortified thus, Eiseley has no difficulty in showing

briefly that Darwin behaved equally badly to Patrick Matthew.

Now much of this may be true, but not for Eiseley’s reasons. In the first place, to interosculate, or osculate, was a well-known word, rarely used because it had become disreputable. The root verb means simply to kiss, and the idea of a toucan (say) kissing a humming-bird was not only hilarious, but associated with the forced and artificial quinary system of the entomologist MacLeay. In this, each genus had five species, each family five genera, and so on; each set of five contained one typical form, two departing from it in opposite directions, and two more departing still further but approaching each other in other characters, so that all five formed a circle. Now the five circles of species making up the five genera of a family could similarly be grouped in a larger circle. Where the circles touched, species of different genera osculated with each other, and so on upwards, with genera, families, orders, and so on, through the whole Animal Kingdom. Nicholas Aylward Vigors, then an ornithologist, in a celebrated paper on the natural affinities of the orders and families of birds (*Trans. Linn. Soc. London*, XIV, 1825) describes the frogmouth *Podargus* as “osculant between the two families” of perching birds and birds of prey (which it is not). In the same paper he remarks of the dodo that being a game bird and winged but flightless, it must belong to the ostrich family, but the presence of a hind-toe allies it to a genus of South American game birds (*Crax*) in the next family. “The bird thus becomes osculant, and forms a strong point of junction between these two conterminous groups”, and so on. Likewise, in an epitome of his classification he remarks of the merganser genus *Mergus* that it “seems to belong to the fourth subfamily, [of ducks] but to be at the extremity of it; in fact to be osculant between the families of *Anatidae* and *Colymbidae*” [the divers, which again, it is not.]

The word was notorious enough for Charles Darwin to use it jocularly in a letter to Henslow in 1832. As Lady Barlow points out in her note to this remark (*Darwin and Henslow*, 1967, page 62, footnote 2) Darwin couples it with MacLeay’s name, and probably got it direct from him, and there is no reason at all to assume that he read it only in Blyth. He could equally well have met it in Vigors and a number of others. Lady Barlow is referred to four times in the book, but not for this

important note. Eiseley's detective work is decidedly amateur; he himself apparently had only met it in Blyth, and he did not have the background of scholarship in the literature of the period to recognise its true status at the time. In his own note (page 242 of the present book, note 69), he says of "interosculate", "The term appears to be used by Blyth and Darwin in the sense of adjoining, not blending.". He could not possibly have phrased it thus if he had actually read any of the quinary literature, or even paused to reflect on the derivation of the term. Whatever metaphysical poets may say, even human beings do not blend while kissing.

Moreover, when one turns to Blyth's actual papers, it must be said that they are disappointing. It is true that, in a young man's fashion, they take up and restate without acknowledgement most of the accepted tenets of the day. They are the work of a young man trying to mark out his own position. If he wanted to make a charge of plagiarism, why did not Eiseley accuse Blyth of plagiarising Erasmus Darwin? Blyth dealt with the variations of plumage in birds more thoroughly than Erasmus did, but nearly all his topics can be found in Erasmus's publications. Where Charles may well have used Blyth's papers was where he used the *Zoonomia* — in his first notebooks to make sure he had brought together all the relevant topics that could bear upon the transmutation of species. Eiseley speaks of the principle of natural selection being known before Charles Darwin to Lyell and to Blyth. In fact, stabilising selection was a commonplace for a hundred years before. Precisely what Blyth had not, as Eiseley has to confess, was any conception of natural selection as causing indefinite departure from a type.

In this respect, Eiseley has seriously misread Blyth. He quotes him (page 58) rightly as appreciating that in the wild, a population of a species that has moved into a new environment may adapt by natural selection on new individual variants to its new circumstances. Blyth goes on "*May not then, a large proportion of what are considered species have descended from a common parentage?*". The italics, however, are Eiseley's (see page 159, where the paper is reprinted), and he clearly takes the remark to mean that for a moment Blyth has glimpsed the production of new species by natural selection. What the rest of Blyth's paper and a very common complaint in the literature of the period show, is that Blyth is protesting that so many newly recognised "species" are merely local varieties produced in this way. Eiseley suggests that Blyth had to reject "this intriguing possibility" because species are not observed to blend with one another. If the possibility had been pointed out to Blyth, he would indeed have given that as a reason for rejecting it; but there is no evidence that he saw the possibility at all. Eiseley later says: (page 66) "In 1837,

however, as has been noted, Blyth showed a few signs of doubt, hastily suppressed, concerning this notion" of the stability of species. This sentence is a choice example of far too much of Eiseley's writing throughout the first part of the book. The atmosphere is electrified for the sake of journalistic excitement out of all proportion to the evidence. Blyth did not suppress anything here, hastily or otherwise. Darwin (page 10) "remained stonily silent" — did he, or just cautiously silent? What is the evidence? He "chose to ignore publicly the papers of Edward Blyth" (page 71) — did he? What was there in them that was not pretty common property? His "eagerness . . . to disclaim any assistance from Lamarck" (page 75) was "little more than the common English

reaction of the time" — it was far more likely to be because he had actually read him.

"A literary critic once made the observation that if Loren Eiseley had lived in the nineteenth century, he would have been a novelist" (preface, page ix). On the evidence of this book he was very much a writer of *romans à thèse*. No, this is not "Vintage Eiseley" (book-jacket); amusing, stimulating, suggestive though it is. He is far better remembered for his *Darwin's Century*. Almost everything that he has written here needs to be done again, much more carefully. □

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Mysterious Stonehenge

Stonehenge and Its Mysteries. By M. Balfour. Pp.189. (Macdonald and Jane's: London, 1979.) £6.95.

ONE could fill a bookcase with the books published on Stonehenge and related topics in the past 10 years. A few are erudite and informative, some are mystical and misleading, and most of the others are popular and pretty. This book is in the last category. It is described by the publishers as "heavily illustrated"; this is almost an understatement, as there are so many photographs, sketches, diagrams and reproductions of works of art, that barely one-third of the book is actual text, even including 9 pages of references and index.

The author is not a professional archaeologist, but a writer and publisher, whose interests lean towards the history of archaeology and the representation of prehistoric sites in art. He has set out the book in six parts: What was it for? In Art and Imagination; The Stones; The Construction of Stonehenge; Twelve local Prehistoric Sites; and Stonehenge and the Public.

Part 1 is the longest and much of it is devoted to a survey of the remarks of early writers on Stonehenge. (Of the 240 references at the end of the book, no fewer than 79 are to works written before 1900.) This part also includes a short account of the astronomical interpretation of Stonehenge, but the astronomical principles are dealt with in such a cursory way that anyone who is not already familiar with them would find the explanation rather difficult to follow. It is a pity that in such a lavishly illustrated book, more informative diagrams were not included, especially when Figures 6 and 11 (both plans of Stonehenge and almost identical) could have been combined into one, and at least two others in this chapter could have been omitted completely

without serious loss. Part 1 concludes with a few words on ley lines and "earth mysteries" and here the author clearly but unconsciously makes the reader aware that he is not writing as a trained scientist.

Part 2 is a fascinating compilation of pictures. Most archaeologists are familiar with Logan's engravings of Stonehenge dating from about 1690, but few readers will have seen reproductions of all the paintings and sketches collected together by Balfour. No doubt cost precluded it, but one would have liked a selection of the paintings reproduced in colour. It would have been most interesting and a useful reminder that the appearance of Stonehenge has changed in recent times, if in a few instances modern photographs showing the stones from the same viewpoints had been set beside the pictures for comparison.

The third part of the book is quite short, barely 3,500 words, and in it the author describes how petrological studies led to the discovery, by H.H. Thomas in 1923, that the origin of the bluestones was in the mountains of South Wales. He discusses how they could have been transported by water and land, the manpower required, and how the stones were dressed. To the reviewer's astonishment, in the middle of this well-written and fairly technical exposition, there is a page of pseudo-scientific gobbledygook, of which this sentence is an example (Page 88): "Accepting that the principles of scientific biofeedback exist, was it once possible for man to transfer energy to and from some stones, set in a particular arrangement — and with establishment of this, in rhythm, to synchronise his visceral and glandular functions so that psychic phenomena could be controlled systematically and at will?".

The section on the construction of Stonehenge takes the form of a chapter within a chapter. After a few pages of introduction, which attempts to set Stonehenge into a cultural background, there is a detailed description of the various phases of construction, closely following

R.J.C. Atkinson's interpretation of the sequence of events. For some reason this is printed in a slightly smaller type and the pages are 'boxed', as though they were a supplement to the main text. This is the most useful part of the book to anyone unacquainted with the subject, and it is enhanced by photographs of old excavations and famous archaeologists. The remainder of the book is something of an anti-climax. Balfour describes twelve 'local prehistoric sites', but without properly linking them to Stonehenge or to the cultural development of the Neolithic and Early Bronze Ages. Indeed, one site (Old Sarum) is not contemporary with Stonehenge by the best part of a thousand years, and the explanation given for its inclusion is that it is on a ley line joining Stonehenge with Salisbury Cathedral!

Part 6 relates to the recent history of

Stonehenge, its changes of ownership, the adverse consequences of its popularity, and controversy over the preservation of the monument. Balfour criticises the Department of the Environment for inept custodianship and puts forward his own proposals for better management of the remains.

In short, this is not a book which will appeal to readers of *Nature*, nor is it one which they would be likely to recommend to their students. It would be appreciated by the interested teenager, and perhaps lead on to more substantial fare. But if you do give it as a Christmas present, don't forget to warn the recipient about page 88.

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Nature and genesis of tumours

The Genesis of Cancer: A Study in the History of Ideas. By L. J. Rather. Pp.262. (Johns Hopkins University Press: Baltimore and London, 1979.) £12.25.

THIS book provides a detailed study of the evolution of scientific ideas on the nature and genesis of tumours. The work is not primarily concerned with aetiology in the sense of formal causation, but rather with concepts relating to the actual body components from which tumours are formed, and the factors determining their gross and microscopic structure. The theories and controversies on cancer current during successive phases in the development of biomedical science are passed in review, and in order to deal adequately with ideas of this kind Dr Rather, professor emeritus of pathology at Stanford University School of Medicine, has of necessity had to survey the long history of scientific thought in general from its earliest beginnings in the Greek classical period. Chapters are therefore devoted to humoral theory from the fifth century BC to 1800 and to tissue theory over the same period, and cover such topics as the movement of blood, the role of lymph, the idea of cancer 'seeds', the theory of fibre structure of tissues, the birth of the 'cellular' theory, the idea of generative membranes, and the germ-layer theory. Each of these subjects is dealt with in minute detail with copious quotations from the writings of the various authors making the original discoveries or contributing to the debates they generated; and as an overall theme, the problems of tumours, tumour structure and tumour growth are slotted in at every stage.

On this broad-ranging yet detailed historical basis some modern ideas are foreshadowed with the flowering of the cell theory following the perfection of the achromatic microscope in the 1830s. This

section of the work may be of interest to general scientific and medical readers outside the ranks of specialists in the history of medicine, for here Dr Rather deals in great detail with Schwann's original theories and their reception, with the concept of tissues built of cells defined in the light of Schwann's work, and with the new contributions to cell theory made by Rokitsky and Virchow in the 1850s and 1860s. The last section of the book brings the story closer to the present time by covering the period 1852 to 1900, considering particularly cytogenetic, embryological and 'blastomal' theories of tumour cell origin; the great debate on the origin of epithelial tumours, and Virchow's connective tissue theory, are also dealt with at length within the setting of the new cellular pathology. A few paragraphs at the end of the book provide a brief opportunity to place the ideas and developments of the nineteenth century in perspective in relation to modern

molecular biology which grew directly from them.

The book is admirably written and provided both with copious notes and a full bibliography; indeed, more than a quarter is devoted to these latter sections which include extensive quotations from the many original sources cited. For the general reader, *The Genesis of Cancer* will perhaps prove excessively specialised, not to say daunting. However, if persevered with, a broad picture of the evolution over many centuries of biological and medical ideas emerges from the fog of minutiae and gives rewarding insights into the way in which our present views on cancer have grown directly from past controversies.

It is perhaps a pity that the disadvantages of a small typeface have been made worse by a tendency to avoid paragraphing, but otherwise the production of the book is good and its presentation attractive. One may perhaps wonder at the spelling of Würzburg as 'Wuerzburg' and the description of the Scots as 'Scotch', but these are minor cavils. It is also strange that Harvey's great contribution should be dismissed on the grounds that '... it is unlikely that he would have made his discovery had he not travelled to Padua for his medical education', when he so obviously indeed chose to travel there for this purpose. In a similar way the contribution of Malpighi likewise fails to receive its due. But apart from these small points, Dr Rather has written a scholarly and useful book which will be of considerable significance for historians of medicine and which may in addition be of interest to medical and scientific readers outside this speciality.

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Physics in a national Canadian laboratory

Physics at the National Research Council of Canada, 1925-52. By W. E. K. Middleton. Pp.238. (Wilfrid Laurier University Press: Waterloo, Canada, 1979.) C\$9.

THIS book tells the story of the rapid growth of research in physics in the National Research Council laboratories in Ottawa before 1953. They began in 1929. Like the National Bureau of Standards in the United States and the National Physical Laboratory in the United Kingdom, they became the home of the national physical standards. They engaged in research also for other government departments and for private industry. By 1939 they were the most important centre of scientific

research in the country.

Major-General A. G. L. McNaughton, who became the President of the NRC in 1935, early recognised that World War II was inevitable. Before it began in 1939 he had already brought some military problems to the laboratories and a year later their work was almost entirely directed to War-time purposes. It included developments in range finding, aerial survey and reconnaissance, aerodynamics, radar, ballistic measurement, gauge calibration, aircraft de-icing, military optical instruments, radiography and other testing of aircraft castings and other military supplies, and many other tasks. Most of this was done by the Physics and Engineering Division and the Divisions that evolved from it. The book does not discuss the War-time research at the NRC in other science than physics, such as on high explosives, poisonous gases, refrigeration of meat during overseas

transportation, and so on.

Dr C. J. Mackenzie was Acting President while McNaughton was on leave to command the Canadian Army overseas. After the War the laboratories returned to peacetime activities and Mackenzie was made President. Some of the scientists were transferred to the new Defence Research Board. Some went to private industry engaged in the development and manufacture of optical, radar and electronic equipment that resulted from their research with NRC. The physicists who remained were mainly in the Physics Division under Gerhart Herzberg and the Applied Physics Division under L. E. Howlett.

By limiting attention mainly to research in Ottawa, Middleton may have left the wrong impression that there was very little other research in physics in NRC laboratories. Almost all nuclear energy research in Canada until 1952 was carried out within the organisation of its Atomic Energy Division. It administered the Montreal Laboratory where the joint UK — USA — Canada nuclear research programme was carried out from 1942 to 1946, and the nuclear research at Chalk River until 1952.

The author reveals the personalities of the scientists while describing their work. I think he is unkind to their first Divisional Director, Dr R. W. Boyle, by dwelling more on his increasing ineffectiveness before he retired in 1946 than his good qualities in earlier years. Some of us who were with him as young scientists when the Division began remember him as a very likable person who encouraged us and did much to create the comradeship, enthusiasm and high morale that inspired the Division.

Because this history ends in 1952 it misses the less happy years when the NRC was unfairly attacked by a Committee of the Senate; the Government then deprived it of some of its responsibilities and has supported it inadequately since. Its critics claimed that its research was predominantly in esoteric fundamental science and that it neglected work of immediate application to problems of national importance. The claim is quite untrue. Dr Middleton's history shows that, although the research included brilliant work in basic science by Gerhart Herzberg and Keith Macdonald, there was far greater activity in applied research and that it was far more strongly supported. It should be read by every Canadian who is deeply interested in national science policy.

The War work was secret at the time. Each scientist knew very little about the activities of others except his close associates. The author has been surprisingly successful in uncovering so much information on the work outside the Optics Section (where he worked) that was buried in a variety of documents and in the memories of the scientists involved. He may fairly be excused for omissions which

members of other Sections might think important. In any case, they will be fascinated in learning about their colleagues' work.

This book should interest students who plan careers in scientific research. It illustrates how research may change in purposes, tools and organisation to meet new demands, that good scientists can adapt to such changes, and that they are often very effective in new fields because they bring to it knowledge acquired in their former specialist fields. It shows that scientists who are well informed in fundamental science can find satisfaction

also in applying it to problems of more obvious and urgent need.

It will have particular interest for Canadian readers because it tells a part of their country's history in the Second World War that is scarcely known. For physicists of other nations it provides an opportunity to compare their own experiences with those of physicists in Canada.

George C. Laurence

George C. Laurence was President of the Atomic Energy Control Board of Canada before he retired, and was a physicist with the National Research Council of Canada for many years.

Darwin the writer

Charles Darwin. By L.R. Stevens. Pp.159. (Twayne Publishers/G.K. Hall: Boston, 1979.) \$8.95.

THIS text in Twayne's English Author's Series by Professor Stevens is devoted to Charles Darwin as a prose writer. A specialist in, and teacher of English, he seeks to integrate Darwin's scientific works with his more discursive biographical writings. The limitation of space to a mere 159 pages, and the even more crippling lack of insight into the range and subtlety of Darwin's natural history seriously prejudice the outcome. Nevertheless, the attitudes of authors such as Carlyle, George Eliot, Matthew Arnold and the Brownings are most informatively set out to reveal an all too frequent incomprehension and, in Carlyle's case, inexcusably arrogant dismissal. There is much perceptive and sympathetic comment and it is relatively infrequently that the author is betrayed into extravagance by the lunatic fringe psychoanalyst usefully particularised in the bibliography.

A chance discovery in the so-called "Edinburgh" notebook (concealed — as are all his quotations from manuscript — under the invariable note "Cambridge Manuscript") coincided with Professor Stevens' illusion that "anxiety and freedom are the two poles between which Darwin's life oscillated" and his consequent reading of Darwin's "assured" as "afraid", to make Darwin's factual note "In Australia I was assured wild dog copulate freely with tame . . ." into "In Australia I was afraid. Wild dog copulates freely with tame . . ." The reference to Australia must be post 'Beagle'. This is but one example of disasters precipitated by a poetic imagination and an insecure grasp of detail. Some few more examples herewith: page 19, *flustra* (*sic*) (as a genus it should have a capital) is not a parasite, the so-called 'ova' are developing larvae of a marine leech, an ectoparasite; page 30, Chagas disease is caused by a protozoan blood parasite conveyed by an infected

blood sucking bug, *not* a bacterium as here implied; page 33, John Herschel, famous son of a famous father (William), wrote the book which inspired Darwin's enthusiasm as well as directing the Observatory at the Cape. He also made a notable collection of Cape bulbous plants.

Professor Stevens' recognition of Darwin's considerable merit as a prose stylist with a highly developed complex use of metaphor should have been backed up by better proof reading. All the notes to "Life and Letters" refer to the two-volume USA edition; the bibliography only gives the three-volume English edition, leaving the ordinary reader completely baffled. It is a good point that (page 95) Darwin "saw more and more wonderful things packed into more and more compressed spaces." Man was seen "dwelling within nature, a completely integrated part of the rhythm and flow of things." Darwin neither courted nor feared a mechanical materialistic view; indeed, the scale ranged from the earthworm to major earth movements as early as 1837. It was not until the last two years of his life that he returned to the manageable scale of the earthworm as earth mover. Professor Stevens (page 102) makes a shrewd counter to Geoffrey West's view of Darwin as the "Anaesthetic Man" by remarking that in the "Descent of Man" Darwin realised "that aesthetics held a crucial and ambiguous place in his argument, and that ambiguity generated in him a lively curiosity, richer in its consequences than a mere one-dimensional utilitarianism can account for."

For some twenty years, Lord Snow's concept of the two cultures and its militant rebuttal by Dr Leavis have confronted the common reader. The peaceful and perceptive middle way chosen by Professor Stevens seems the more profitable route for today, and reveals Darwin's creative imagination to both scientist and humanist. Scientists have much to learn from this study.

Sydney Smith

Sydney Smith was formerly Lecturer in Zoology at Cambridge University, and is now an Emeritus Fellow of St Catherine's College, Cambridge, UK.

Determinant of contemporary society?

Nuclear Power for Beginners, Including the Alternatives. By Stephen Croall and Kaiaanders Sempler. Pp.176. (Beginners Books/Writers and Readers Publishing Cooperative: London, 1979.) Hardback £3.50; paperback £1.80.

"NUCLEAR power is complicated, but not that complicated. The basics are fairly simple. Anyway it's not just a question of techniques or economics. It's about *the kind of society* we want for ourselves and future generations".

It really is strange the way nuclear power is inflated into the position of being a determinant of the evolution of contemporary society. It is seen by those for it and against it as having an almost mythical power to influence the totality of social activity; far beyond that of much more pervasive influences such as the telephone or the motor industry.

This, of course, is not the issue addressed in this book. The twin hypotheses on which it rests are that nuclear power *does* determine the future of society; and for the worse. Accepting these for the moment it is interesting to see the way the case is developed. The format is that of a lurid comic book. There are plenty of death's heads, bombs, and King Kong multinational companies trampling the populous underfoot. Civil nuclear power is tied up in the same package as nuclear warfare, imperialism, reckless and wanton abuse of the environment, and a suicidal disregard for the future of the human race.

It is very well done. The text and pictures are humorous and compelling; the reader is pulled through the book quite effortlessly. The amount of information conveyed is quite remarkable, as is the level of its sophistication. As an exercise in communication it succeeds very well indeed. What it communicates is, of course, another question entirely. There is not the slightest attempt to be balanced or objective. And there is the crux of the nuclear argument. It is too seldom about nuclear power.

The alternative vision of society here is based on the proposition that the 'Soft Energy Path' of Amory Lovins is a proven alternative. China of the Cultural Revolution is said to have "come closest to the soft energy model". If true, so much the worse for soft energy. The details of the Chinese experience in the 1966-76 decade are emerging. They show that behind the rhetoric of participation, people's democracy, and self-help was a reality of anarchic arbitration, oppression, and waste of resources which brought the country to the brink of collapse. If the country was a "display window for alternative ecologically viable techniques"

then it showed they bring a crop of problems as grave as those they were meant to solve.

It is not to say that there is no valid point to be drawn from this book; the pity is that the nuclear debate has to be conducted so often on the basis of conflicting rhetorics and hyperbolic pretensions. For every fantasy on the anti-nuclear side there is one on the pro-nuclear side. Evasions, half-truths, and downright lies litter that side of the debate too.

In the full picture of the world's energy needs nuclear power is not very important. But it can have its uses. It should take its place in the array of choice facing society — to be assessed on its merits, and dangers, against the alternatives. But in the context of all the pressures which create the "kind of society" we are passing on to future generations nuclear power is a minor

element. The passionate arguments that occur both for and against nuclear power are a diversion from much more serious topics.

For its merits as a piece of propaganda, and its enrichment of the fantasy world of nuclear power this book deserves its place on the bookshelf with all those other books of dreams which began with a vision of electricity so cheap it would not be worth the expense of metering. The point is becoming clearer every day: both sides expected too much from nuclear power. It is neither as bad as those who were driven to produce this book feared, nor as great a boon as those in favour of it believed it was going to be. It is time the argument moved on.

Gerald Foley

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Electricity harnessed

Electricity from Glass: The History of the Frictional Electrical Machines, 1600-1850. By W.D. Hackman. Pp.310. (Sijthoff-Noordhoff: The Netherlands, 1978.) \$44.50.

THIS is a very welcome book — for what it provides, what it suggests and what it makes possible.

What it provides is a classification scheme and history of frictional electrostatic machines, devices which constituted the first important electrical invention. Together with the Leyden jar, they made possible, by the 1840s, production, storage and discharge of substantial quantities of electricity. Hackman's classification is by critical physical factors: globe versus cylinder versus plate; horizontal versus vertical axis; single versus multiple elements. Such an approach is convenient for the person who has a machine which needs to be identified; and it is reasonable historically, as many of the features were seen as improvements which were adopted and which superseded previous designs.

The suggestion is that these machines were important, as surely they were. With the more impressive effects now available, electricity moved out of its infancy into the realm of fully fledged science. Experiments no longer consisted of rubbing glass tubes and picking up bits of paper. They included sparks several inches long and discharges strong enough to knock a man senseless. Entirely new categories of tests could be performed, with new theoretical structures to match them. One clear result was the pioneering work of Benjamin Franklin, who began his investigations just as the new apparatus was being introduced. He was thus confronted almost immediately with

the need to explain sparks and shocks and induction, and he bypassed the more subtle phenomenon of electrostatic attraction which had dominated the thinking of his contemporaries who had come on the scene slightly earlier. The former line of attack proved fruitful, the latter was a dead end. Furthermore, the impressiveness of the effects made them valuable as educational tools, increasing the interest of the public and of students in electrical phenomena. Investigators were quick to seize the opportunity, and some were giving accounts of impressive demonstrations.

The book makes possible the beginnings of a systematic catalogue of a surprisingly large number of objects, and it is reasonable to presume that by bringing together information about a substantial portion of them we could gain considerable insight into the interactions among instrument makers, and the interdependence of theory and practice during the early years of an important discipline.

For this reason, it would have been a great help if the number of photographs had been increased — by a factor of two or three at least, over the thirty-four presently included — and if more attention had been paid to the quality of reproduction. Also desirable — though this would probably demand a new research project — would be the sort of details one usually finds in museum catalogues: critical dimensions, chemical analyses and descriptions of construction materials.

The book is certain to encourage many people to look at their electrical machines with new understanding. Inevitably, it will stimulate the accumulation of further information and lead us into a better understanding of the origins of electricity as an experimental science.

Bernard S. Finn

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No new solutions

Nature's Price: The Economics of Mother Earth. By W. van Dieren and M.G.W. Hummelinck. Pp.193. (Marion Boyars: London and Boston, 1979.) £3.50.

THE authors of this book have tried to relate economics to ecology. They attempt to set a price on the natural assets of the Earth, the Sun and wind, the soil and water, on minerals and, particularly, on wildlife. Their thesis is that by underestimating the cost of these resources and the way they are wasted in the effort to achieve our so-called progress, we are entering into some very bad bargains. They try to shock us into reversing our attempts to secure unlimited industrial growth and ever-increasing agricultural productivity, which they say are leading rapidly to the destruction of the globe on which we live.

As a conservationist, and one who wishes to see the world's flora and fauna and our wild environment protected for the enjoyment of posterity, I hoped to find some new solutions to our problems, and to be taught new arguments to forward our aims. Unfortunately this book makes no striking new contributions to its subject, and it contains a good deal of ecological nonsense. The authors' aims are admirable, but they seldom hit the right target.

The reader has surely the right to expect that a book dealing with *The Economics of Mother Earth* contains accurate financial information, so that costs can be properly compared. Many pages are devoted to pest control, spelling out the dangers of chemical pesticides as against the advantages of non-chemical controls. Chemicals are shown (in my opinion rightly) to have potential ecological dangers, and to be expensive. The value of a pest-eating bird is more difficult to quantify. However, the figures for chemicals are seriously incorrect. We are told that "the world spent £16,000 million (\$30,000 million) on chemical pesticides in 1973"; in fact the world bill was only a tenth of this figure. Even in 1978, when chemical prices had risen threefold following the increase in oil prices, the sum involved was still only about half that suggested by van Dieren and Hummelinck. This error affects much of the discussion.

Elsewhere there are other misleading statements. In a discussion on the danger of producing strains of insects resistant to insecticides (a very real problem, I agree) we are told that "malaria is occurring on a greater scale than ever before. In Sri Lanka, the number of malaria victims has increased from a few hundred to two millions in the last fifteen years". This shocking increase has indeed taken place in Ceylon, but not because the vector cannot be controlled. This is an instance of the success of the extreme conservationists,

who caused the use of DDT to be stopped, so malaria once again became epidemic. Of course insecticide resistance is a real problem, as stressed in the recent report on Agricultural Pollution of the UK Royal Commission on Environmental Pollution, but in most countries where malaria control continues, the disease decreases and insecticides continue to be one of the important weapons ensuring this decrease.

The sections dealing with agriculture are equally disappointing. All the old misinformation about the inefficiency of modern mechanised farming is repeated. We have the dogmatic statement that "harvests cannot keep on growing by putting more money (artificial fertiliser, machines, pesticides) into the ground", and we are assured repeatedly that a diverse population of wild animals will ensure better and more stable cropping. Would that these things were true; the life of the conservationist would then be easy. These authors can never have seen a patch of mealies grown in an area of the richest ecological diversity that has been visited by an elephant. Though bad arable farming can have disastrous results on the soil and

the environment in Britain and in the Netherlands (from which country this book originates) good intensive farming is steadily improving soil fertility; but most wildlife (except the soil flora and fauna, which flourishes) plays little part in the scenario.

This is all very sad. These authors have clearly the best of intentions. They recognise the very real dangers of overpopulation and of uncontrolled resource depletion, and make some not very original suggestions regarding their prevention. But once more they make us realise that, if we wish to preserve wildlife, we must come clean and give the right reasons, which are that we like to have it around, and that we should not pretend that they have such great economic value. In Britain we may regret the extinction of the bear and the wolf, but their reintroduction, though interesting to the enthusiast, would hardly increase the productivity of our farmers.

Kenneth Mellanby

Kenneth Mellanby was Director of Monks Wood Experimental Station from 1961-74.

Phenomena of superconductivity

Magnetic Flux Structures in Superconductors. By R.P. Huebener. Pp.259. (Springer: Berlin, Heidelberg and New York, 1979.) DM59; \$32.50.

THIS book originates from the conference of the same title held at Argonne National Laboratory, 1973, and from subsequent graduate lecture courses. The presentation of material in the book assumes a familiarity with the phenomena of superconductivity although, perhaps reflecting its origins in a lecture course, results which are probably already well known, such as the quantisation of flux, are briefly derived again. The mathematics is everywhere very simple: the pattern throughout is to outline the physical ideas, derive the fundamental equation and then where appropriate to quote others' results, possibly even in simplified form. The emphasis is on exposing the reader to the basic physical phenomena and the diverse experimental techniques which are used to investigate them. Many references (over 500) are given throughout the text to enable those so inclined to follow up any of the topics.

The first half of the book is devoted to the static case. The different intermediate state structures of Type I superconductors are discussed and there are a number of photographs which illustrate the richness of the varieties observed in different materials and geometries. Discussion of the mixed state of Type II superconductors follows a brief introduction to the

Ginsburg-Landau theory. Interactions between one vortex line and another and with surfaces are calculated. The vortex lattices, defects and correlations with crystal lattices are described and illustrated. This section ends with a review of nine techniques of investigating these flux structures.

The second half of the book looks at the dynamics of flux motion; because of the variety of effects and their complexity more use is made of referring the reader to original papers.

The motion of flux due to transport currents, changes in the magnetic field and temperature gradients are discussed phenomenologically; and the Hall effect, Ettinghausen and Peltier effects, the Josephson relation, transport entropy as well as instabilities and force free configurations are outlined. The time-dependent Ginsburg-Landau theory is carefully introduced and some results from it are given. The mechanisms for flux pinning has a short chapter as does flux creep and flux jumps. The techniques for studying the individual and collective motions of vortices are reviewed, and a chapter is devoted to electrical noise power analysis. The final chapter discusses the current-induced resistive state with the emphasis on phenomena observed in thin films.

This book will be useful to researchers and to people involved in engineering aspects of superconductivity, particularly of small low field devices.

J.G.M. Armitage

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